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ACTIVATION OF THE RIBOSOMAL RNA GENES IN ERYTHROCYTE NUCLEI OF
XENOPUS LAEVIS

by

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Submitted for the Degree of Doctor of Philosophy.

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SUMMARY

Xenopus laevis erythrocyte nuclei have been used as a source of inactive ribosomal RNA genes to study the regulation of gene activity during early embryogenesis. Addition of an oocyte extract to the erythrocyte nuclear transcription assay brings about transcription by RNA polymerase I. This is not seen if an egg extract is used even though the amounts of RNA polymerase I are the same in both preparations. The oocyte-treated nuclei synthesize ribosomal RNA as defined by RNA-DNA hybridizations and sucrose gradient separation techniques.

The active component of the oocyte extract has been semipurified to a single peak by Sephadex G100 column chromatography and DEAE cellulose salt elution. Its presence and absence during oogenesis and early embryogenesis parallels the transcription of the ribosomal genes in vivo at these stages. Within the oocyte itself, the active component is located exclusively in the germinal vesicle.

The structure of the ribosomal genes in treated nuclei has been studied by DNase I digestion. The ribosomal genes in erythrocyte nuclei are insensitive to DNase I, but treatment by an oocyte extract or egg extract plus the semipurified active component causes the entire gene repeat to become DNase I sensitive. An egg extract alone has no effect. The semipurified active component by itself causes only the 5' end of the transcribed sequence to become DNase I sensitive. The component therefore appears to act on the non-transcribed ribosomal genes of the erythrocyte nucleus to alter their chromatin conformation to a more DNase I sensitive one which permits RNA polymerase I transcription.

The DNase I sensitivity of histone H4, globin, tRNA and 5Soc RNA genes was also studied. The extracts had no effect on the DNase I sensitivity of any of these genes, but those genes transcribed by RNA polymerase III are DNase I sensitive whatever their transcriptional state.

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CONTENTS

	Page
Summary	ii
Contents	iii
Index of Figures	vi
Index of Tables	xii
Acknowledgments	xiv
Declaration	xv
Abbreviations	xvi
 CHAPTER I; Introduction.	 1
Transcription During Oogenesis of <u>Xenopus laevis</u> .	1
Transcription during Early Embryogenesis of <u>Xenopus laevis</u> .	6
Control of Transcription during Oogenesis and Embryogenesis.	9
Changes in Chromatin Structure as a Means of Regulating Transcription.	15
Methods for Studying the Control of Transcription.	21
 CHAPTER II; Materials and Methods.	 23
Materials.	23
Methods.	28
 CHAPTER III; Transcription in Erythrocyte Nuclei and the	
Effect of Cellular Extracts.	43
Preparation of Erythrocyte Nuclei.	44
Salt Optima for Erythrocyte Nuclear Transcription.	45
RNA Polymerase Activity in Erythrocyte Nuclei.	47

Effect of Oocyte and Egg Extracts on Erythrocyte Transcription.	50
RNA Polymerase Levels in Erythrocyte Nuclei Treated with Egg and Oocyte Extracts.	54
RNA Polymerase Levels in Oocyte and Egg Extracts.	58
Effect of RNA Polymerase I on Erythrocyte Nuclear Transcription.	60
Calcium and Transcription by Erythrocyte Nuclei.	62
Change in Size of Erythrocyte Nuclei by Oocyte Extract.	62
Transcription in Liver Nuclei.	64
CHAPTER IV: Location and Occurrence of the Ribosomal	
Activator during Oogenesis and Early Embryogenesis.	65
RNA Transcription during Oogenesis.	65
RNA Polymerase Levels During Oogenesis.	68
Presence of the Ribosomal Gene Activator During Oogenesis.	68
Location of the Ribosomal Gene Activator within the Oocyte.	70
Presence of Ribosomal Gene Activator during Early Embryogenesis.	71
RNA Polymerase Levels during Embryogenesis.	72
Presence of the Ribosomal Gene Activator during Embryogenesis.	73
CHAPTER V; Purification by Column Chromatography of the	
Component of Oocytes which Stimulates RNA Polymerase I.	77
Sephadex G100 Column Chromatography of Oocyte Extracts.	77
Sephadex G100 Column Chromatography of Germinal Vesicle and Enucleated Oocyte Extracts.	82
Sephadex G100 Column Chromatography of Egg Extract.	83

Sephadex G100 Column Chromatography of Embryo Extracts.	84
DEAE Cellulose Chromatography of Oocyte Extracts.	86
DEAE Cellulose Salt Elution of Egg and Neurula Extracts.	88
Visualization on Acrylamide Gels of the Oocyte Stimulatory Protein, Fr-18.	89
Migration of Fr-18 into Erythrocyte Nuclei.	90
Effect of Fr-18 on Transcription by Erythrocyte Nuclei.	91
CHAPTER VI; Fidelity of Transcription by RNA Polymerase I on Erythrocyte Nuclear Chromatin.	93
Hybridization to the Ribosomal RNA Probe, pX1101.	95
Hybridization to Restriction Endonuclease Fragments of Ribosomal Plasmid, pX1r108.	97
Fractionation of the RNA on Sucrose Gradients.	99
CHAPTER VII; The DNase I Sensitivity of Erythrocyte Ribosomal DNA.	101
Effect of DNase I on Erythrocyte Nuclei.	104
Hybridization Conditions.	104
DNase I Sensitivity of the Ribosomal RNA Genes in Liver and Erythrocyte Nuclei.	105
DNase I Sensitivity of Other Genes.	115
DNase I Sensitivity of Genes Transcribed by RNA Polymerase II.	116
The DNase I Sensitivity of Genes Transcribed by RNA Polymerase III.	118
CHAPTER VIII; Conclusions.	124
REFERENCES.	128

INDEX OF FIGURES

	Facing Page
CHAPTER III	
3.1 Nuclear Lysis During Incubation.	45
3.2 Salt Optima.	46
3.3 Specific Activity of ^3H -UTP in the Transcription Assay.	46
3.4 Triphosphate Optimum.	46
3.5 Test for Degradation of RNA Product of Transcription by Erythrocyte Nuclei.	47
3.6 Number of Erythrocyte Nuclei in the Transcription Assay.	47
3.7 Inhibition of Transcription in Erythrocyte Nuclei by α -amanitin	48
3.8 Relative Levels of RNA Polymerases I, II, and III in Erythrocyte Nuclei.	48
3.9 Effect of Oocyte and Egg Extracts on Transcription by Erythrocyte Nuclei.	51
3.10 Optimal Concentration of KCl During Preincubation.	52
3.11 Preincubation Time.	52
3.12 Relative Levels of the Three RNA Polymerases in Extract-Treated Erythrocyte Nuclei.	55
3.13 Inhibition of Transcription by Erythrocyte Nuclei by Rifamycin AF/013.	56
3.14 Effect of Sarkosyl on Transcription by Erythrocyte Nuclei.	57
3.15 RNA Polymerase Activity in an Oocyte Extract.	59
3.16 RNA Polymerase Levels in Oocyte and Egg Extracts.	59
3.17 Transcription on Erythrocyte Chromatin or poly[d(A-T)] Produced by Added, Semipurified RNA Polymerases.	60
3.18 Effect of Increasing the Concentration of Oocyte Extract in the Transcription Assay.	60

3.19 RNA Polymerase Levels in Oocyte Extracts.	61
3.20 RNA Polymerase Levels in Erythrocyte Nuclei Treated with Oocyte Extracts.	61
3.21 Addition of Extra RNA Polymerase I to Erythrocyte Nuclei Supplemented with Oocyte Extract.	61
3.22 Dilution of Oocyte Extract by Column Buffer and Egg Extract	62
3.23 Effect of Calcium and EGTA on Transcription by Erythrocyte Nuclei.	62
3.24 Change in Dimensions of Erythrocyte Nuclei on Incubation with Oocyte Extract.	63
3.25 Transcription by <u>Xenopus laevis</u> Liver Nuclei.	64
3.26 Relative Levels of the Three RNA Polymerases in Liver Nuclei.	64

CHAPTER IV

4.1 Relative Amounts of the Three RNA Polymerases During Oogenesis.	68
4.2 Effect of Oocyte Extracts from Different Stages of Oogenesis on RNA Polymerase I Transcription in Erythrocyte Nuclei.	69
4.3 Effect of Different Stage Oocyte Extracts on RNA Polymerase II and III Transcription in Erythrocyte Nuclei.	70
4.4 Relative Amounts of the Three RNA Polymerases in the Germinal Vesicle and Cytoplasm of the Oocyte.	70
4.5 Relative Amounts of RNA Polymerases I, II and III Activities in Erythrocyte Nuclei Treated with Extracts from Enucleated Oocytes and Germinal Vesicles.	71

4.6 Levels of the Three RNA polymerases During Early Embryogenesis.	72
4.7 Effect on Erythrocyte Nuclear Transcription of Extracts from Different Stage Embryos.	73
4.8 Relative Levels of the Three RNA Polymerases in Erythrocyte Nuclei Preincubated with Embryo Extracts.	73
4.9 RNA Polymerase I Activity in Erythrocyte Nuclei Treated with Embryo Extracts.	74

CHAPTER V

5.1 Fractionation by Sephadex G100 of an Oocyte Extract.	78
5.2 Oocyte G100 Column Assay with Erythrocyte Nuclei and Egg Extract.	79
5.3 Oocyte G100 Column Assay with Erythrocyte Nuclei, Egg Extract and Fr-18.	80
5.4 Oocyte G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.	80
5.5 Oocyte G100 Column Assay with poly[d(A-T)].	81
5.6 DNA Nicking Activity in the Oocyte G100 Column Fractions.	82
5.7 Heat Inactivation of Fr-18.	82
5.8 Fractionation by Sephadex G100 of an Enucleated Oocyte Extract.	83
5.9 Fractionation by Sephadex G100 of an Oocyte Germinal Vesicle Extract.	83
5.10 Fractionation by Sephadex G100 of an Egg Extract.	84
5.11 Egg G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.	84
5.12 Fractionation by Sephadex G100 of an Early Cleavage Extract.	84

5.13 Fractionation by Sephadex G100 of a Gastrula Extract.	84
5.14 Fractionation by Sephadex G100 of a Neurula Extract.	84
5.15 Early Cleavage G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.	85
5.16 Gastrula G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.	85
5.17 Neurula G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.	85
5.18 Heat Inactivation of "Fr-18" from Neurula G100 Column.	85
5.19 Fractionation by Sephadex G100 of a Combined Oocyte and Neurula Extract.	85
5.20 Effect of NaCl on Transcription by Erythrocyte Nuclei.	86
5.21 Fractionation by DEAE cellulose of an Oocyte Extract.	87
5.22 Heat Inactivation of the Active Component Separated on a DEAE cellulose Column.	87
5.23 Oocyte DEAE cellulose Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.	87
5.24 Fractionation by DEAE cellulose of Fr-18.	87
5.25 Fractionation by DEAE cellulose of an Egg Extract.	88
5.26 Fractionation by DEAE cellulose of Pooled Fractions 17-19 from an Egg G100 Column.	88
5.27 Fractionation by DEAE cellulose of a Neurula Extract.	88
5.28 Fractionation by DEAE cellulose of Fractions 17-19 from a Neurula G100 Column.	88
5.29 Acrylamide Gel Analysis of Oocyte G100 Column Fractions.	89
5.30 Acrylamide Gel Analysis of Proteins in Fractions 17-19 of Oocyte, Egg and Neurula G100 Columns.	89
5.31 Migration of Oocyte Proteins into Erythrocyte Nuclei.	90
5.32 Migration of Oocyte Proteins into Erythrocyte Nuclei.	91

5.33 Relative Levels of the Three RNA Polymerases in Nuclei Preincubated with Egg Extract and Fr-18.	92
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CHAPTER VI

6.1 Restriction Map of the Ribosomal Gene Repeat of <u>Xenopus laevis</u> .	95
6.2 Optimal Time for RNA-DNA Hybridization.	95
6.3 Restriction Map of rDNA Plasmid pXlr108.	98
6.4 Transcription of the rDNA Non-Transcribed Spacer in Erythrocyte Nuclei Pre-treated with an Oocyte Extract.	98
6.5 Sucrose Gradient Fractionation of H-RNA Isolated from Erythrocyte Nuclei Preincubated with an Oocyte Extract.	99

CHAPTER VII

7.1 Digestion of Erythrocyte DNA by DNase I.	104
7.2 Optimum Time for DNA-DNA Hybridizations.	105
7.3 DNase I Sensitivity of the Ribosomal RNA genes in Erythrocyte and Liver Nuclei.	105
7.4 DNase I Sensitivity of the rDNA in the Erythrocyte Nuclei Preincubated with an Oocyte or Egg Extract.	106
7.5 DNase I Sensitivity of the rDNA in the Erythrocyte Nuclei Preincubated with an Oocyte or Egg Extract.	106
7.6 DNase I Sensitivity of the rDNA in the Erythrocyte Nuclei Preincubated with an Oocyte or Egg Extract.	106
7.7 Effect of a DNase I Hypersensitive Site.	107
7.8 Presence or Absence of an Hypersensitive Site in rDNA.	108

7.9 DNase I Sensitivity of rDNA in Preincubated Erythrocyte Nuclei where Transcription was Inhibited.	109
7.10 Restriction Map of <u>Xenopus laevis</u> Ribosomal DNA.	110
7.11 DNase I Sensitivity of Parts of the rRNA Gene in Erythrocyte Nuclei.	111
7.12 DNase I Sensitivity of Parts of the rRNA Gene in Erythrocyte Nuclei.	111
7.13 DNase I Sensitivity of Parts of the rRNA Gene in Erythrocyte Nuclei.	111
7.14 DNase I Sensitivity of Parts of the rRNA Gene in Erythrocyte Nuclei.	111
7.15 Differential DNase Sensitivity of the rDNA from Erythrocyte Nuclei.	112
7.16 DNase I Sensitivity of the Globin Genes in <u>X.laevis</u> Erythrocyte and Liver Nuclei.	116
7.17 DNase I Sensitivity of the Globin Genes in <u>X.laevis</u> Erythrocyte and Liver Nuclei.	116
7.18 DNase I Sensitivity of the Histone H4 Genes in <u>X.laevis</u> Erythrocyte and Liver Nuclei.	117
7.19 DNase I Sensitivity of the Histone H4 Genes in <u>X.laevis</u> Erythrocyte and Liver Nuclei.	117
7.20 DNase I Sensitivity of the 5Soc RNA Genes in <u>X.laevis</u> Erythrocyte and Liver Nuclei.	118
7.21 DNase I Sensitivity of the 5Soc RNA Genes in <u>X.laevis</u> Erythrocyte and Liver Nuclei.	118
7.22 DNase I Sensitivity of a selection of tRNA Genes in <u>X.laevis</u> Erythrocyte and Liver Nuclei.	120
7.23 DNase I Sensitivity of a selection of tRNA Genes in <u>X.laevis</u> Erythrocyte and Liver Nuclei.	120

INDEX OF TABLES

	Facing Page
CHAPTER III	
3.1 Transcriptional Activity of Stored Erythrocyte Nuclei.	45
3.2 Effect on Transcription in Erythrocyte Nuclei of Preincubation with Protein Extracts.	52
3.3 Effect on Erythrocyte Nuclear Transcription of Preincubation with Protein Extracts.	53
3.4 Effect of an Extract of Defolliculated Oocytes on Transcription by Erythrocyte Nuclei.	53
3.5 Inhibition of Transcription by Actinomycin D.	54
3.6 Necessity of Active RNA Polymerases During Preincubation.	57
3.7 RNA Polymerase Levels in Oocyte and Egg Extracts.	59
3.8 Transcription by Liver Nuclei Incubated with Protein Extracts.	64
CHAPTER IV	
4.1 RNA Polymerase Activity in Extracts of Oocytes of Different Stages.	68
4.2 RNA Polymerase I Activity in Erythrocyte Nuclei Preincubated with a Pre-vitellogenic Oocyte Extract.	69
4.3 RNA Polymerase I Activity in Erythrocyte Nuclei Preincubated with Extracts from Oocytes of Different Stages.	70
4.4 RNA Polymerase Activity in Erythrocyte Nuclei Preincubated With Extracts of Oocyte GVs and Cytoplasm.	71

CHAPTER V

5.1 Effect of Increasing Centrifuge Speed on Oocyte and Egg Extracts.	77
5.2 Trypsin Inactivation of Fr-18.	82
5.3 Trypsin Inactivation of Active Component from a Neurula G100 Column.	85
5.4 Inactivation of Active Component from an Oocyte DEAE Column.	87
5.5 Effect of Rifamycin AF/013 and Actinomycin D on Transcription by Erythrocyte Nuclei Preincubated with Egg Extract and Fr-18.	92
5.6 Time of Addition of Fr-18.	92

CHAPTER VI

6.1 Presence of rRNA Transcripts from Erythrocyte Nuclei Preincubated with Various Extracts.	95
6.2 Amount of Transcription of the 18S and 28S Regions by Treated Erythrocyte Nuclei.	96

CHAPTER VII

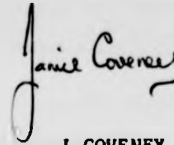
7.1 DNase I Sensitivity of Regions of the Ribosomal RNA Gene Repeat.	112
7.2 DNase I Sensitivity of Various Genes in Erythrocyte and Liver Nuclei.	120

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DECLARATION

I hereby declare that this thesis was composed by myself and has not been submitted in any other previous application for a degree. I carried out all the work described here, except for the DNA extraction step and sample loading for the gel shown in Figure 5.6, which was done by Prof. H.R.Woodland. All sources of information have been acknowledged by means of reference.

A handwritten signature in cursive script, reading "Janice Coveney". The signature is written in dark ink on a light background.

J. COVENEY

ABBREVIATIONS

ATP	adenosine-5'-triphosphate
CTP	cytidine-5'-triphosphate
GTP	guanosine-5'-triphosphate
UTP	uridine-5'-triphosphate
XTP	ribonucleotide-5'-triphosphate
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
mRNA	messenger RNA
hnRNA	heteronuclear RNA
tRNA	transfer RNA
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate
dTTP	thymidine-5'-triphosphate
dNTP	2'-deoxy-ribonucleotide-5'-triphosphate
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
rDNA	DNA sequences encoding the rRNA genes
poly[d(A-T)]	poly-deoxy-adenylic-deoxy-thymidylic acid
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene-[bis(oxy-ethylenenitrile)] tetraacetic acid
DMSO	dimethyl sulphoxide
SDS	sodium dodecyl sulphate
PPO	2,5-diphenyloxazole
POPOP	1,4-di-2(5-phenyloxazolyl)benzene
UV	ultra violet
OD	optical density
cpm	counts per minute

CHAPTER I; INTRODUCTION.

Transcription During Oogenesis of *Xenopus laevis*.

There are three classes of RNA polymerase in eukaryotes, I, II, and III (eg. Roeder et al, 1970; Tocchini-Valentini and Crippa, 1970; Keding et al, 1972; Roeder, 1974a; Schwartz et al, 1974; Weil and Blatti, 1975; 1976; Hager et al, 1977; these polymerases have also been called A, B, and C, respectively). The three RNA polymerases have different templates, physical properties and are likely to have different control mechanisms. Their transcription can easily be distinguished since they have different sensitivities to the fungal toxin, α -amanitin (Jacob et al, 1970). Thus RNA polymerase I, which transcribes the ribosomal RNA genes, is insensitive to α -amanitin, whilst mRNA and hnRNA synthesis by RNA polymerase II is inhibited by only 1 μ g/ml α -amanitin. RNA polymerase III transcription of the 4S and 5S RNA genes is inhibited by 100 μ g/ml α -amanitin.

Xenopus laevis was an early species in which RNA polymerase in development was studied, because material is readily available in bulk. Transcription in sea urchins and *Drosophila* is also well characterized, the former due to the ease of obtaining and handling large numbers of eggs and embryos, and the latter, even though the handling is more difficult, does have the advantage of a wide range of well defined mutants not available in either *Xenopus* or sea urchin. The polymerases of eggs of the moth *Manduca sexta* have also been well characterized (Kastern et al, 1981).

Whilst the oocytes of Xenopus laevis make RNA at all stages, the molecular species synthesized vary from stage to stage (Mairy and Denis, 1971; Ford, 1971), although the relative amounts of the three RNA polymerases remain the same (Roeder, 1974b). The small, pre-vitellogenic oocyte synthesizes a preponderance of 4S (tRNA) and 5S RNA (Mairy and Denis, 1971; 1972; Thomas, 1974), such that the rRNA 40S precursor molecules are outnumbered by about 100:1 (Ford, 1971). In Xenopus there are several subtypes of 5S RNA genes, the major type being oocyte specific (5Sooc). They are a highly reiterated gene family, consisting of about 20,000 copies/haploid genome (Brown et al., 1971). There are two other subtypes, a minor oogenetic 5S set (1300 copies/haploid genome; Brown et al., 1977; Peterson et al., 1980) and a somatic specific set (5Ssom; 400 copies/haploid genome; Wegnez et al., 1972; Brownlee et al., 1972; Ford and Southern, 1973; Peterson et al., 1980). All 5S sequences are transcribed during oogenesis (Wegnez et al., 1972; Denis and Wegnez, 1977), but whilst the 5Sooc RNA molecules are stored in 7S or 42S ribonucleoprotein particles until needed (Ford, 1971; Picard et al., 1980), the 5Ssom RNA molecules do not accumulate (Denis and Wegnez, 1977). Associated with each 5Sooc gene is a pseudogene which lacks the final 19 bp at the 3'end of the gene (Jacq et al., 1977). In vivo, this pseudogene is thought not to be transcribed, but this has been observed on injection of 5S DNA into Xenopus oocytes (Miller and Melton, 1981). The synthesis of 4S and 5S RNA continues throughout oogenesis, although after vitellogenesis begins and rRNA synthesis starts, it assumes a smaller fraction of the total oogenetic RNA (Ford, 1971; Mairy and Denis, 1971; 1972; Thomas, 1974)

Transcription by RNA polymerase II is also seen in pre-vitellogenic oocytes (Rosbash and Ford, 1974; Darnborough and Ford, 1976; Ruderman and Pardue, 1977; Golden et al, 1980). One consequence of this is that there is enough histone H3 mRNA to account for that seen in the mature oocyte present in these small oocytes (van Dongen et al, 1981). The same is true for the ribosomal protein mRNAs (Pierandrei-Amaldi et al, 1982). This is despite the fact that the lampbrush chromosome structure is not seen until after vitellogenesis. Lampbrush chromosomes are the site of intense transcriptional activity, about 100-fold the rate seen in normal somatic cells (Anderson and Smith, 1978), but whether the RNA transcribed from the lampbrush loops is degraded soon after synthesis, or whether it is required to maintain the oocyte mRNA pool is not known. This pool could have a turn over of relatively stable mRNAs with a half-life of 2 to 3 months, or the mRNA synthesized in pre-vitellogenic oocytes could have a short half life.

As vitellogenin is processed and deposited in the oocyte, so rRNA begins to be transcribed in mass (Mairy and Denis, 1971; Brown and Littna, 1964a, b). All three RNA polymerases are present in approximately equal activities throughout oogenesis (Roeder, 1974b), so it is not lack of RNA polymerase I that is responsible for the lack of rRNA synthesis seen in the pre-vitellogenic oocyte. Neither is it lack of template. The rRNA synthesized during oogenesis is transcribed from extrachromosomal nucleoli (Gall, 1968). These 1400 to 1500 nucleoli (Perkowska et al, 1968) are produced at least in part via a rolling circle mechanism (Brown and Blackler, 1972; Hourcade et al, 1973; Rochaix et al, 1974) from the chromosomal rDNA, and give about a 1000-fold increase in the number of ribosomal rRNA

genes (Dawid et al, 1970), from the 1880 chromosomal copies to 1.5 to 2.5 million copies. This amplified rDNA is synthesized during pachytene (MacGregor, 1968; Kalt and Gall, 1974; van Gansen and Schram, 1974), so it is present in even the earliest stages of oogenesis.

The ribosomal genes in Xenopus, as in many other species, are repeated many times. Those of Dictyostelium discoideum are located with the 5S genes forming extrachromosomal palindromic dimers (Cockburn et al, 1976; Maizels, 1976), whilst those of Xenopus, Drosophila and mammals are chromosomal tandem repeats, unlinked to the 5S genes (Wellauer and Dawid, 1977; 1979; Boseley et al, 1978; Glover and Hogness, 1977; Pellegrini et al, 1977; Cory and Adams, 1977). There is a basic repeat unit, in the pattern: gene-nontranscribed spacer-gene, where the spacer region is of a variable length. The non-transcribed spacer in Xenopus laevis is well characterized (Boseley et al, 1979), and can be divided into four sections. The first, at the 3' end of the transcribed sequences, is invariant in length (500 bp) and is not internally repetitive. It is followed by an internally repetitive region of variable length (97 bp repeats) and a "Bam Island" (320 bp) which is non-repetitive, centred on a Bam HI site. The final section consists of two related repetitive regions (60/81 bp repeats) separated by the second Bam Island. Both Bam Islands are similar in sequence to each other, and appear to be reduplications of the promoter region in the first 20 bp of the 40S rRNA precursor molecule (Moss and Birnstiel, 1979).

There is a further complication in the case of the

Drosophila ribosomal genes; some of the 28S rRNA gene sequences are interrupted by a non-coding segment (Long and Dawid, 1980). These genes are not transcribed (Long and Dawid, 1979) although the initiation sequences are identical in both types of rRNA genes (Long et al, 1981).

By amplifying the rDNA, the time taken to transcribe the 4 ug rRNA found in the mature oocyte is decreased to a manageable length. The other ribosomal RNA species - 5S RNA - which is synthesized earlier than the 40S rRNA, is stored in ribonucleoprotein particles until required (Ford, 1971; Picard et al, 1980). These two ribosome components are therefore controlled by quite differing mechanisms, not only are they transcribed by different RNA polymerases, but the stage during oogenesis when they are transcribed is also different. The problem of accumulating the two types of rRNA is thus solved in two ways. In one case by amplification of the genes and transcription over part of oogenesis, in the other by transcription from a lesser number of unamplified genes over a more extended period. The final number of ribosomes produced is large (¹²10¹²; Perkowska et al, 1968), there are enough ribosomes to keep the embryo alive, without further rRNA synthesis, until the feeding tadpole stage (Brown and Gurdon, 1964). The Xenopus oocyte, unlike that of Drosophila and many other insects, synthesizes all of the rRNA that is found in the unfertilized egg. There is no involvement from surrounding follicle cells, unlike the nurse cells of insects that produce most of the rRNA (Hughes and Berry, 1970). This could be a reflection of the shorter oogenesis of insects as compared to Amphibia.

Transcription during Early Embryogenesis of *Xenopus laevis*.

The unfertilized egg and early cleavage embryo of *Xenopus laevis* are inactive with respect to RNA synthesis, this is despite there being no loss in the measurable activity, and no change in the relative amounts of the RNA polymerases (Roeder et al, 1970; Roeder, 1974b). However translation of the mRNAs synthesized during oogenesis continues in the egg and early cleavage embryos. Thus the stored histone and actin mRNAs are made use of in the lag period between the end of oogenesis and when new mRNA species begin to be transcribed in the blastula stage embryo (Adamson and Woodland, 1974; Ballantine et al, 1979).

There has been much controversy concerning the time when transcription recommences, since this system does not naturally lend itself to investigation. The embryo is impermeable to labelled RNA precursors, and there is a large pool of cold RNAs that were synthesized during oogenesis that may mask a small quantity of de novo RNA synthesis; but the major problem is the very few nuclei that are available at the early stages of embryogenesis. One solution to this problem is to use autoradiography to study early transcription (Bachvarova and Davidson, 1966; Gurdon and Woodland, 1969; Newport and Kirschner, 1982a)

No transcription has been measured in the earliest embryonic stages (Shiohawa et al, 1981a; Newport and Kirschner, 1982a). 4S and mRNA synthesis have been reported to restart in blastulae or earlier (Brown and Littna, 1964b). There would appear to be a developmental transition in midblastula embryos which brings

about transcription (Newport and Kirschner, 1982a). This is initiated by the change in the ratio of nucleus to cytoplasm and exerts its effect on the genomic DNA, as well as injected, non-homologous cloned genes (Newport and Kirschner, 1982b).

The time of onset of rRNA synthesis is not as clear. Originally this was placed at a later stage than other RNA species, in gastrulae (Brown and Littna, 1964a, b). More recent work indicates that rRNA synthesis can be measured in mid to late blastula stage embryos (Shiokawa et al., 1981a, b). The new rRNA transcription is from the chromosomal rDNA, rather than the extrachromosomal rDNA which appears to be dispersed during oocyte maturation, fertilization and early cleavage divisions (Busby and Reeder, 1982). rRNA synthesis is not uniform over the entire embryo, in neurula embryos the presumptive endoderm does not synthesize as much rRNA as the remainder of the embryonic tissues (Woodland and Gurdon, 1968; Misumi et al., 1980). This disparity is gradually decreased, such that the entire embryo synthesizes RNA at about the same rate by the swimming tadpole stage. Whether the rRNA synthesis seen in blastulae and gastrulae is significant, and whether because of the low rate of synthesis these genes could be termed 'active' remains a moot point (Davidson, 1976). Certainly the rate of rRNA synthesis in the early developmental stages is not as great as that seen during oogenesis. The actual amount of rRNA synthesized in blastulae, as measured by Shiokawa et al. (1981b), is very small, 0.02 pg rRNA/cell/hour, and this increases to 0.12 pg rRNA/cell/hour in neurula embryos. Not all cells in these embryos synthesize rRNA (Woodland and Gurdon, 1968; Misumi et al., 1980), so if these figures are adjusted to account for only those cells with nucleoli, a figure of 0.2 pg rRNA/nucleolated cell/hour is

reached. The apparent increase in rate of rRNA synthesis between blastulae and neurulae is therefore a result of an increasing percentage of the embryonic cells having nucleoli. The early stage embryos have very few cells with nucleoli visible, whilst 80% of the neurulae cells have nucleoli. This increase in the number of cells with nucleoli is paralleled by the decrease in the percentage of free RNA polymerase molecules and the concomitant increase in the number of RNA polymerases on the chromatin (Thomas et al., 1980). The amount of rRNA transcribed in these embryonic cells is 40% of the maximum capable in rapidly dividing somatic cells, where the rate has been calculated at 0.5 pg rRNA/cell/hour (Perkowska et al., 1968). It would appear, therefore, that the ribosomal genes in those embryonic cells that have nucleoli can be described as active, although they are not being transcribed at their full potential. This does not make them 'inactive' however. Those cells without nucleoli could be classed as inactive with respect to their rRNA transcription. The value for the rate of transcription of the rRNA genes in embryo cells with nucleoli (40%) can be compared to the number of rRNA genes necessary to allow the developing embryo to survive. Anucleolate mutants that lack all the rRNA genes and therefore transcribe no new rRNA during embryogenesis do not survive past the swimming tadpole stage, by which time the maternal ribosome pool is exhausted (Brown and Gurdon, 1964). Animals that have intermediate levels of rDNA are known (Knowland and Miller, 1970; Miller and Knowland, 1970), and those with 45% or 60% of the wild type gene number survive, but those that have only 35% of the wild type gene number do not survive much longer than those with no rDNA (Miller and Knowland, 1972). This would indicate that the embryo with a normal rDNA content can only transcribe its rRNA genes at

about 40% of the maximum rate; in other words, the wild type and the viable rDNA deletion mutants have a certain amount of redundancy, but the non-viable mutants fall below the critical ribosomal gene content.

Control of Transcription during Oogenesis and Embryogenesis.

As previously mentioned, although the relative levels of the three RNA polymerases remain the same during oogenesis (Roeder, 1974b), the amount of synthesis they achieve in the cell varies enormously. Thus the activity of the respective gene classes does not seem to be dependent on the amount of each RNA polymerase. The same is true for the early embryonic stages. The amount of RNA polymerase remains the same as that found in oocytes, but in the early stages no RNA synthesis is seen, and once transcription recommences different genes in some classes are transcribed. RNA polymerase I still transcribes rDNA, but during oogenesis it is confined to the extrachromosomal nucleoli rather than the chromosomal sequences. The 5S RNA genes transcribed by RNA polymerase III are also different. In somatic cells it is the 5S_{som} genes that are transcribed, the 5S_{ooc} genes are not used (Wegnez et al, 1972; Brownlee et al, 1972; Ford and Southern, 1973). As the embryo develops and tissues differentiate the sequences transcribed by RNA polymerase II also change.

Early work on rRNA synthesis during embryogenesis (Yamana and Shiohawa, 1966; Shiohawa and Yamana, 1967; Wada et al, 1968) indicated that these genes were regulated by an inhibitor molecule. The assay, which involved incubating dissociated neurula cells with extracts from earlier developmental stages, showed that blastular extracts could inhibit rRNA synthesis in

the intact neurula cells. A similar inhibitory factor was isolated from stage 6 oocytes that specifically inhibited rRNA synthesis in stage 4 oocytes (Crippa, 1970). However, Crampton and Woodland (1979a, b), using a different assay, containing isolated cultured cell nuclei, showed that rRNA synthesis is regulated by a series of activating molecules present in oocyte and neurula extracts. These two systems are quite different in character; while the first concentrates on whole cells, the second is based upon isolated nuclei. It is therefore possible that both revealed molecules controlling rRNA synthesis in vivo.

Whereas the control of rRNA synthesis is not well understood, the same is not true of 5S gene transcription. The products of these two gene families are present in equimolar amounts in ribosomes, but the two genes are transcribed by different RNA polymerases and under different control mechanisms. It is easily seen that the transcription of the 5S genes is not dependent on the transcription of the rRNA genes since 5S RNA synthesis is seen before rRNA synthesis during oogenesis (Brown and Littna, 1964a, b; Ford, 1971; Mairy and Denis, 1971; 1972; Thomas, 1974) and during embryogenesis (Brown and Littna, 1964b; Miller, 1973; 1974; Shiohawa et al, 1981a, b). The lack of dependence between rRNA transcription and 5S RNA transcription can also be shown since anucleolate or nu/nu⁻ Xenopus laevis embryos which lack the ribosomal RNA genes (Brown and Gurdon, 1964) transcribe the 5S genes efficiently (Miller, 1973; 1974). Although rRNA transcription is not linked to 5S RNA transcription, it does have some influence over the translation of the ribosomal protein mRNA. Anucleolate mutants, although they transcribe 5S RNA genes to the same extent as their wild type

siblings, do not translate the ribosomal protein mRNA (Pierandrei-Amaldi et al., 1982).

An oocyte nuclear extract can accurately transcribe 5S DNA cloned in a plasmid, although some vector sequences were also transcribed. When an egg extract is substituted for the oocyte nuclear extract, all the transcription is non-specific (Birkenmeier et al., 1978; Ng et al., 1979). This formed the basis for a transcription assay; various oocyte fractions were added to the basic non-specific egg + plasmid assay and accurate transcription sought (Engelke et al., 1980). A 37000 dalton protein, called TFI_{IIA}, was identified which is specific for 5S_{om} and 5S_{oc} genes, but not the tRNA genes, which are also transcribed by RNA polymerase III. It binds to the internal control region of the 5S genes, between bases 45-96 independently of the RNA polymerase III molecules (Sakonju et al., 1980; Bogenhagen et al., 1980). This region is necessary for transcription to occur when 5S DNA is injected into Xenopus oocytes or used in an in vitro transcription assay (Wormington et al., 1981). The areas necessary for transcription in the oocyte system and for binding of the 37 kd protein are co-extensive (Sakonju et al., 1981). The protein may well be involved in feedback regulation, as it also binds to the 5S RNA gene product to form the 7S ribonucleoprotein complex (Pelham and Brown, 1980; Honda and Roeder, 1980) of immature oocytes, it is also responsible for the formation of a stable transcriptional complex (Bogenhagen et al., 1982; Gottesfeld and Bloomer, 1982). There also seems to be an immunologically similar, but not identical, molecule present in somatic cells of 39,000 daltons molecular weight (Pelham et al., 1981). The presence of the two 5S regulatory proteins does not, however,

solve the problem of 5Sooc and 5Ssom gene control, since a somatic cell transcription assay will transcribe both sets of genes with comparable efficiencies (Pelham et al, 1981).

Oocyte injection transcription assays and systems such as those described for 5S DNA have also been used to identify control regions neighbouring genes transcribed by RNA polymerase II (Luse and Roeder, 1980; Wasylyk et al, 1980; Luse et al, 1981). By deleting and rearranging flanking regions before assaying the ability of the genes to initiate and support transcription, as well as by the comparison of flanking regions between different genes, it has been possible to identify several important sequences. Apart from the -TATA- or -ATA- sequence usually situated 25 to 30 bp upstream from the 5' end of the gene (Grosveld et al, 1981; Tsai et al, 1981; Mathis and Chambon, 1981), other upstream sites have also been implicated, for example, the sequence -CAAT- at about 80 bp upstream (Benoist et al, 1980; Grosveld et al, 1982). An enhancing element, like those found in DNA viruses, for example SV40 (Benoist and Chambon, 1981; Gruss et al, 1981, Tyndall et al, 1981), that would stimulate transcription at an upstream promoter site could also be a possibility. The SV40 enhancing sequence is located between 113 and 257 bp from the transcription initiation site. It is a 72 bp repeated unit that can be ligated to eukaryotic cloned genes to increase their transcription by up to 2 fold when assayed (Moreau et al, 1981). Also associated with the control of these genes are sites downstream from the transcribed sequences, the termination site and signals for polyadenylation (-AATAAA-, Proudfoot and Brownlee, 1976; Hofer et al, 1982). With genes transcribed by RNA polymerase II, where the final product is a protein and not the primary

transcript, the further possibility of translational control arises. This possibility is made use of by X.laevis, for example in the case of histones and actins, where the pool of mRNA synthesized during oogenesis is under-utilized until embryogenesis.

Transcription systems for RNA polymerase I have been more difficult to set up (Grummt, 1981). One reason for this is the that accurate transcription of a cloned rDNA template is dependent on the template and RNA polymerase I molecules being homologous (Grummt et al, 1982). There appear to be no -TATA- or -ATA- sequences, but the sequence -AGGTA- has been implicated in initiation (Sollner-Webb and Reeder, 1979; Long et al, 1981; Miller and Sollner-Webb, 1981). However, the actual site of initiation in mouse rDNA is unclear (Miller and Sollner-Webb, 1981). The initiation site of the Xenopus rRNA genes (Sollner-Webb and Reeder, 1979; Sollner-Webb and McKnight, 1982) is complicated by the presence of a reduplicated promoter (Moss and Birnstiel, 1979) within the non-transcribed spacer that can initiate transcription. However, it appears to be located between -145 bp and +16 bp (Moss, 1982), that is upstream from the initiation point. The promoter for RNA polymerase I, therefore, is more like an RNA polymerase II promoter than that of RNA polymerase III in its upstream location.

The standard assays outlined above have the disadvantage that for many of them the template is in the form of naked DNA, whereas normally in vivo the DNA is associated with a multitude of histone and non-histone proteins. Therefore many transcription assays have been designed about isolated nuclei

(eg. Marzluff et al, 1973). This does give rise to the problem, that for genes transcribed by RNA polymerase II at least, the amount of transcription is small, because the gene copy number is very low, although this is not an insurmountable problem. Isolated nuclei continue to synthesize the same RNA species as before isolation, and a variety of newly initiated RNA types have been reported, including 5S RNA (Marzluff et al, 1973), immunoglobulin kappa light chain mRNA (Smith and Huang, 1976) and Drosophila heat shock protein mRNA (Miller and Elgin, 1981).

Korn and Gurdon (1981) injected erythrocyte nuclei into Xenopus oocytes and looked for 5S RNA synthesis. The chromatin of Xenopus erythrocyte nuclei is highly condensed and the only transcription seen is elongation by the remaining RNA polymerase II molecules (Hentschell and Tata, 1978). The injected erythrocyte nuclei did synthesize 5S RNA, but in the oocytes of many females it was of the somatic type, as expected from somatic cells. Preincubation of the nuclei with the 5S regulatory protein (Engelke et al, 1980) did not alter this, although removing the non-histone proteins from the nuclei did bring about 5Sooc RNA synthesis. The inactivity of the 5Sooc genes injected in this system is at the level of the gene unit itself. Cutting the chromatin into fragments of this size does not alter their inability to be transcribed (Gurdon et al, 1982). A few female Xenopus did produce oocytes that would cause erythrocyte nuclei to transcribe both sorts of 5S RNA genes. but the difference between the two types of oocytes is unknown.

Crampton and Woodland (1979a, b) showed that rRNA synthesis in cell culture nuclei could be increased by the addition of a

range of oocyte factors. Unfortunately, because nuclei were used that were already actively transcribing their rDNA it was not clear exactly how these oocyte factors worked, and whether they 'activate' genes from a former inactive state (assuming that there is a mixed population of active and inactive rRNA genes in a given nucleus), or increase the transcription on already active genes.

In a different system, studying insect polymerase II genes, Craine and Kornberg (1981) succeeded in altering the transcriptional state of nuclei from Drosophila embryos. By incubating nuclei isolated from non-heat-shocked embryos with cytoplasmic extracts from heat-shocked embryos, they showed that certain of the heat-shock protein genes become more accessible to transcription by E.coli RNA polymerase. However, this region is not normally transcribed, and it does not show that this 'factor' can also bring about de novo transcription by the Drosophila RNA polymerase II molecules, or that the change in accessibility of the E.coli RNA polymerase is reflected in a change in chromatin structure.

Changes in Chromatin Structure as a Means of Regulating Transcription.

Chromatin structure in itself has been studied as a mechanism for the control of gene activity. The genomic DNA is bound to a range of histone and non-histone proteins. The nucleosome core - an octomer of two molecules of each of histones H2A, H2B, H3 and H4 - is encircled by about 200 bp of DNA, which after a short "linker" of about 15 bp DNA, continues on around another nucleosome. Histone H1 is associated with this "linker"

DNA, although its exact function is unclear. It may be concerned with locking the DNA about the nucleosome. In some tissues, histone H1 is replaced by an H1 variant, for example, histone H₀ found in sperm nuclei and histone H5 in mature erythrocyte nuclei. In chicken erythrocytes, which are transcriptionally almost inert, the H1 molecules are gradually replaced by histone H5. It gradually replaces the H1 molecules as erythropoiesis proceeds and transcription gradually ceases (Appels et al., 1972; Appels and Wells, 1972; Billett and Hindley, 1972; Sotirov and Johns, 1972).

The position of the nucleosomes upon the DNA has been postulated as a method of regulating transcription. Nucleosomes can either be in random positions on the DNA, or have a fixed location on a given stretch of DNA, or have a limited number of possible locations. Experiments on viral systems have shown that the origin of replication of SV40 and Polyoma virus DNA are nucleosome-free (Varshavsky et al., 1979; Saragosti et al., 1980; Scott and Wigmore, 1978). This presumably aids replication of the viral genome during infection. The case in other phyla though is far from clear. Early work gave no decisive results and was taken as an indication of random nucleosome phasing. More recent work favours a set of non-random nucleosome positions in a variety of genes and species. The occurrence of a non-random nucleosome phasing appears to be favoured by those genes that are being transcribed. Drosophila histone and 5S genes are packaged in a non-random way (Samal et al., 1981; Louis et al., 1980), as are the chicken embryo tRNA(Lys-2) genes. However, transcribed tRNA genes in Xenopus laevis do have a regular, but random, relationship between DNA sequence and nucleosome position, whilst non-transcribed tRNA genes are in one major

phase (Bryan et al., 1981). The nucleosome arrangement for 5S RNA genes of Xenopus laevis is also non-random, in one of four alternative phases, but this phasing is present for both transcribed and non-transcribed sequences (Cottesfeld and Bloomer, 1980).

Apart from the primary coiling of the DNA about the nucleosomes, the chromatin is further folded back on itself several times. This coiling has been cited as the basis of another possible mechanism for gene control (Wasylyk and Chambon, 1979). A gene that is tightly folded would be inaccessible to RNA polymerase molecules and therefore "inactive", whilst a gene that is "active" and being transcribed would be in a more "open" chromatin configuration. However, it could be that the more open configuration is brought about by the transcription and not the more open structure allowing transcription to take place. In either case, such active genes should be more readily accessible to nucleases than those tightly folded and inactive sequences.

Several nucleases have been used to distinguish between the active and inactive sequences within a nucleus, the most widely used is DNase I although micrococcal nuclease and DNase II also achieve this purpose (Reeves and Jones, 1976; Bloom and Anderson, 1978; Wood and Felsenfeld, 1982; Larsen and Weintraub, 1982). Weintraub and Groudine (1976) showed that DNase I could distinguish between transcribed and non-transcribed gene sequences. Chicken globin genes were preferentially digested in erythrocytes, but not in fibroblast or brain cells. This relationship was also shown for the chicken ovalbumin gene (Garel and Axel, 1976); those cells that normally synthesize ovalbumin showed increased sensitivity to DNase I as compared to those

cells that do not transcribe this gene. Since these initial experiments, this facet of DNase I sensitivity has been extended to many more gene systems, and further levels of sensitivity have been described. For the most part, a gene that is being transcribed or is potentially able to be transcribed, is more sensitive than the same gene sequence that is not going to be transcribed. The sensitive domain encompasses the transcribed region, but may also extend for several kb either side of the gene. However, the area about the transcribed region, although more sensitive than the surrounding bulk DNA tends to be less sensitive than the actual transcribed sequences (Stadler et al, 1980)

Also associated with transcribed genes are ultra-sensitive spots of DNase I sensitive chromatin. These may be only a few bases long, or extend for up to 50 bp. They are normally only found in the flanking regions of transcribing genes, but a few exceptions occur in both cases. For example, there are DNase I hypersensitive sites located 300 bp from the 5' end of the Drosophila heat-shock protein genes 83, 28, 23, 26, and 22 whatever the transcriptional state of the genes (Wu et al, 1979 b; Wu, 1980; Keene et al, 1981), although further sites appear when the cells are heat-shocked. One of the hypersensitive sites associated with the glue protein gene Sgs4 of Drosophila is located 30 bp within the transcribed sequence (Shermoen and Beckendorf, 1982), and there are two hypersensitive sites associated with the mouse β globin gene (from erythroleukemia cells) located within the transcribed sequence (Hofer et al, 1982). All other hot spots appear to be outside the transcribed region, although not all are in such close proximity as those of

the Drosophila heat-shock protein genes; for example the site associated with the chicken conalbumin gene is about 1 kb from the 3' end of the gene (Kuo et al., 1979). At least in the Polyoma viral system, the location of the DNase I hypersensitive site is not determined by the nucleotide sequence of the DNA at the hypersensitive site (Herbomel et al., 1981), but perhaps by a DNA sequence upstream from this site.

The hypersensitive sites about the 5' flanking region of the Drosophila glue protein gene Sgs4 at -330, -405 and -480 (Shermoen and Beckendorf, 1982) seem to be associated with sequences that are necessary for transcription of the gene in vivo (Shermoen and Beckendorf, 1982; Muskavitch and Hogness, 1982). These sites are well removed from the 'typical' RNA polymerase II transcription signals at -25 to -30 (-TATA-) and -80 bp (-CAAT-). One of the hypersensitive sites within the mouse β globin gene (Hofer et al., 1982) is in the vicinity of the cap site, and is more prominent in chromatin from induced cells.

The cause of the different chromatin structure in untranscribed and transcribed genes is not clear. The HMG proteins 14 and 17 (Goodwin et al., 1973) are associated exclusively with transcribed sequences (Levy et al., 1979; Weisbrod et al., 1980; Weisbrod and Weintraub, 1981), but it is unlikely that these actually cause the chromatin conformational change (Gazit et al., 1980), although they may be responsible for fixing or maintaining the new chromatin structure. Some Drosophila heat-shock protein genes seem to have their chromatin structure modified in some indeterminate way by a protein (Craine and Kornberg, 1981).

Methylation and demethylation has also been called upon as a trigger for gene activation. In some instances the correlation between the activity of a given gene and the degree to which the cytosine residues are methylated is quite clear (McGhee and Ginder, 1979; Shen and Maniatis, 1980; Compere and Palmiter, 1981). However, lack of methylation per se is not a prerequisite for gene transcription. That is, transcription may still occur if a sequence is methylated (McKeon et al., 1982). The rRNA genes of Xenopus laevis are a case in point. In somatic cell nuclei, although most of the gene repeat is heavily methylated there are two sites of undermethylation in the non-transcribed spacer region (Bird and Southern, 1978). The extra extrachromosomal rDNA in oocytes is also of this pattern, whilst the chromosomal rDNA of oocyte and sperm nuclei are heavily methylated (Bird et al., 1981). It would appear that early in development at or preceding the point at which rRNA transcription recommences, these sites within the non-transcribed spacer become demethylated. The rDNA in the closely related species X.borealis is undermethylated at these sites throughout its life cycle (Macleod and Bird; 1982). In laevis x borealis hybrids the methylation state at the swimming tadpole stage is the same in both maternal and paternal rDNA sequences, but only the laevis rDNA is DNase I sensitive and only these sequences are transcribed. So although demethylation of the gene sequence to be transcribed, or a site near to it may be a prerequisite for transcription to occur, it probably is not the signal for that gene to be transcribed.

Methods for Studying the Control of Transcription.

A good transcription system designed to look at transcriptional control should be as close as possible to the in vivo conditions, whilst being relatively simple in its components. The control of transcription during oogenesis and early embryogenesis of Xenopus laevis is, with the exception of 5S RNA genes, still largely unexplained. Most transcription assays contain recombinant DNA as the template; this has the definite advantage that the system is very simple, but the disadvantage that the DNA is not in the natural chromatin form. In isolated cell culture nuclei, although the genes are associated with the chromatin proteins, they are actively transcribing a whole range of genes, many of which are also transcribed during oogenesis and embryogenesis. The erythrocytes of X.laevis are nucleated, but the chromatin is highly condensed and very little transcription occurs (Maclean et al., 1973; Hentschell and Tata, 1978). It therefore makes a very useful tool to study ribosomal RNA transcription since it is a near-natural source of inactive ribosomal genes.

It is not clear whether rRNA transcription is controlled by an inhibitor (Yamana and Shiohawa, 1966; Shiohawa and Yamana, 1967; Wada et al., 1968), or an activator (Crampton and Woodland, 1979a, b) molecule. Although the use of erythrocyte nuclei will not detect an inhibitor molecule, it might at least detect any activator. Erythrocyte nuclei can be induced to transcribe RNA above their normal rate by injection into Xenopus oocytes (Korn and Gurdon, 1981), and to divide when injected into Xenopus eggs (Brun, 1978), although in this case the "embryo" never proceeds further than the early gastrula

stage. However, these particular systems have disadvantages when trying to distinguish the active component and therefore incubation with oocyte protein extracts is the preferred method of assay. The development and use of this system is the subject of this thesis.

CHAPTER II; MATERIALS AND METHODS.

1. Materials.

Most reagents used were AnalaR grade from BDH. Exceptions and their origins are listed below.

Agar Aids.

Gluteraldehyde, electron microscopy grade.

Amersham International.

[5-³H] uridine 5' triphosphate, ammonium salt, 10-30 Ci/mmol.

deoxy[5-³H] cytidine 5' triphosphate, ammonium salt,
15-30 Ci/mmol.

L-[³⁵S] methionine, 600 Ci/mmol.

deoxy 5'-[α-³²P] triphosphate, triethylammonium salt,
2000-3000 Ci/mmol.

Boehringer Mannheim.

α-amanitin

proteinase K EC 3.4.21.14

poly[d(A-T)]

Kornberg DNA polymerase I EC 2.7.7.7

ATP, CTP, GTP, UTP.

Bio.Rad Laboratories Ltd.

Acrylamide

Fisons Scientific App. Ltd.

Bromophenol Blue (BPB)

Kodak.

N,N'-methylene bisacrylamide

Millipore Corporation.

1.4 cm diameter nitrocellulose filters, pore size 0.45 μ m.

New England Biolabs, Inc.

Restriction Enzymes Bam HI, Eco RI, Hind III.

The reaction conditions used were those included by Biolabs with each batch of enzyme.

Pharmacia Fine Chemicals AB.

Sephadex G100

Ficoll 400

Schleider and Schull.

Nitrocellulose filters, pore size 0.45 μ m.

Schwarz/Mann, Inc.

RNase-free Sucrose

Sigma London Chemical Company Ltd.

Agarose Type II

Trypsin

Trypsin Inhibitor

Dithiothreitol (DTT)

RNase A EC 3.1.27.5

TI RNase EC 3.1.27.3

Actinomycin D

Bovine Serum Albumin (BSA)

Cytochrome c

Blue Dextran

Ovalbumin

Yeast tRNA

dATP, dCTP, dGTP, dTTP

Polyvinylpyrrolidone (PVP)

DNase I from bovine pancreas EC 3.1.21.1

Dithiothreitol (DTT)

Ethyl-m-aminobenzoate (MS222)

Lysozyme EC 3.2.1.17

South African Snake Farm, Fish Hoek, South Africa.

Mature Xenopus laevis

These and their progeny were reared as described by Gurdon and Woodland (1975).

Whatman Chemical Separation Ltd.

DEAE Cellulose

Filter paper No 54

Worthington Biochemical Corp.

RNase-free DNase I EC 3.1.21.1

Plasmid DNA

- pXl101 A gift from Dr A. Colman. It contains a single copy of the X.laevis ribosomal RNA gene repeat (10.75 kb Hind III fragment) inserted into pMB9. As constructed by Dr R. Reeder. See Figure 6.1.
- pXlr108 A gift from Dr A. Colman. It contains the 6.27 kb Eco RI fragment of the X.laevis rDNA repeat inserted into pCRI (Boseley et al, 1978). See Figures 6.1 and 7.10.
- pXlr212 A gift from Dr A. Colman. It contains the 4.48 kb Eco RI fragment of the X.laevis rDNA repeat inserted into pCRI (Boseley et al, 1979). See Figure 7.10.
- pXlr14 A gift from Dr A. Colman. It is a subclone of pXlr108 containing the 1.57 kb Pst I fragment. As constructed by Dr B.E.H. Maden. See Figure 7.10.
- C13 A gift from Dr R.W. Old. It contains about 300 bp of X. laevis globin cDNA inserted into pCRI.
- pcXlH4W1 A gift from Dr P.C. Turner. It contains 382 bp of X. laevis histone H4 cDNA inserted into pAT153 (Turner and Woodland, 1982).
- pXlo31 A gift from Dr A. Colman. It contains a single 660 bp repeat of the X.laevis 5Soc RNA gene, pseudogene and spacer, inserted into pMB9 (Federoff and Brown, 1978).

λt210 A gift from Dr A. Colman. It contains two copies of tRNA-Met1, and a single copy of the tRNA genes for phenylalanine, asparagine, alanine, leucine, lysine, and tyrosine. This is a 3.18 kb genomic DNA fragment inserted into λ598 vector (Clarkson et al, 1978; Bryan et al, 1981).

C13, pcX1H4W1, pX1o31, pX1r14 and λt210 were received as DNA preparations. pX1101, pX1r108 and pX1r212 were received as plasmid-containing bacteria. These were grown and the plasmid DNA extracted as described in the Methods section.

Semipurified X.laevis RNA polymerases were prepared by Prof. H.R. Woodland following the method of Roeder (1974), being a 40% ammonium sulphate precipitate from whole Xenopus laevis ovary. kinase labelled ³²P-18S and 28S rRNAs were also prepared by Prof H.R. Woodland. 2.5 µg 18S and 28S rRNA purified from a sucrose gradient were heat denatured (80°C, 3 minutes) and kinase labelled with 27 µCi ³²P-ATP. This gave ³²P-18S rRNA at 8.5×10^5 cpm/µg and ³²P-28S rRNA at 6.4×10^5 cpm/µg.

The rDNA fragment L-108 (2.18kb cut from pX1r108 by a Bam HI x ECO RI double digest) was a gift from Dr. P. Boseley.

2. Methods.

Preparation of Erythrocyte Nuclei from *Xenopus laevis*.

Homogenization Buffer (H buffer).

0.3 M sucrose (RNase-free), 2 mM Magnesium Acetate, 3 mM Calcium chloride, 10 mM Tris-HCl pH 8.0, 0.1% Triton-X100, 0.5 mM Dithiothreitol.

Centrifugation Buffer (C buffer).

2 M sucrose (RNase-free), 5 mM magnesium acetate, 10 mM Tris-HCl pH 8.0, 0.5 mM dithiothreitol.

Resuspension Buffer (RB).

25% glycerol, 5 mM magnesium acetate, 50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 5 mM DTT.

Calcium and Magnesium-free Barth-X.

Modified Barth solution containing 58 mM NaCl, 1 mM KCl, 24 mM NaHCO₃, 15 mM HEPES.

Nuclei were prepared according to the method of Marzluff et al (1973). A large female *Xenopus laevis* was anaesthetized using 0.1% ethyl-m-aminobenzoate (MS222) and the blood collected by cardiac puncture using a heparin rinsed syringe and diluted into Ca/Mg-free Barth-X plus 0.1 mg/ml heparin. The erythrocytes were washed several times in Ca/Mg-free Barth-X by centrifugation (MSE 6L, 8 x 50 ml rotor, 2,000 rpm for 10 minutes at 4°C) and resuspension to remove all the plasma and white blood cells. The clean erythrocyte pellet was taken up in H buffer and left on ice for 5 minutes before being homogenized in a loose fitting Teflon-glass homogenizer so that all the cells

were lysed. An equal volume of C buffer was added and mixed well. 5 ml aliquots of this nuclear suspension were layered over 8 ml of C buffer in a 15 ml Corex tube. After centrifugation (MSE 6L, 8 x 50 ml rotor, 3,500 rpm for 60 minutes at 4°C) the sucrose solutions were removed carefully and the pelleted nuclei gently resuspended in RB to a final concentration of 2×10^7 nuclei/ml. The nuclei were stored in small aliquots at -70°C.

Preparation of Liver Nuclei from *Xenopus laevis*.

Homogenization Medium (HM).

10 mM Tris-HCl pH 7.4, 10 mM sodium chloride, 1.5 mM magnesium chloride, 50% glycerol (w/v).

The procedure used was a modification of that of Schribler and Weber (1974), the liver from a mature *Xenopus laevis* female was roughly chopped and washed in HM. The pieces were homogenized in a Teflon-glass homogenizer in 4 volumes of HM buffer plus 0.1% Triton-X100 on ice. The homogenate was strained through sterile muslin, briefly spun (MSE 6L, 8 x 50 ml rotor, 2000 rpm for 5 minutes at 4°C) to pellet the nuclei and any unlysed cells. This pellet was rehomogenized on ice in HM plus 0.1% Triton-X100 and centrifuged again. The pellet was further washed 3 times with HM only, and after the final spin the nuclei were taken up in the standard resuspension buffer and stored at -70°C. Although this leaves the nuclei slightly contaminated with pigment granules, further cleaning spins were not carried out to prevent loss of transcriptional activity.

Nuclear Transcription Assay.

The basic 100 μ l assay contained 50 μ l of erythrocyte nuclei in RB plus KCl, MnCl and ribonucleotide-triphosphates at optimal concentrations (Chapter III), such that the final mix was; 10^6 nuclei in 12.5% glycerol, 2.5 mM magnesium acetate, 25 mM Tris-HCl pH 8.0, 2.5 mM DTT, 0.05 mM EDTA, 120 mM KCl, 1 mM MnCl₂, 0.4 mM ATP, CTP and GTP, 0.02 mM UTP and 4 μ Ci ³H-UTP (10-30 Ci/mole). The nuclei were incubated at 25°C and duplicate 10 μ l or 20 μ l samples spotted onto 2 cm pieces of Whatman 54 filter paper at the required times. The filters were dropped into ice-cold 5% TCA, 1% tetrasodium pyrophosphate. Filters pooled in this way were washed three times in TCA/pyrophosphate on ice, twice with ethanol and once with acetone before being dried and the TCA precipitable cpm determined (Toluene-PPO-POPOP scintillant, Packard scintillation counter). These were expressed as pmoles UMP incorporated per 10^6 nuclei.

To preincubate the nuclei with an oocyte, egg or embryo extract, the standard 100 μ l assay contained; 10^6 nuclei, 40 μ l extract, 0.5 x RB, 50 mM KCl, 1 mM MnCl₂, and 0.02 mM ATP, CTP, GTP and UTP. The nuclei were incubated for 60 minutes, then either spun down gently (Eppendorf Microfuge, for the minimum possible time) and taken up in the basic assay mix, or supplemented to bring the salt concentrations etc to those of the standard assay. The nuclei were incubated again at 25°C for 30 minutes, samples taken and processed as above.

RNA Transcription Assay.

Identical conditions to the nuclear transcription assay were used, but the template was poly[d(A-T)]. A standard 50 μ l assay contained 20 μ l of the extract to be tested, 2 μ g poly[d(A-T)], 0.5 x RB, 120 mM KCl, 1 mM MnCl_2 , 0.4 mM ATP, CTP, and GTP, 0.02 mM UTP and 2 $\mu\text{Ci}^3\text{H}$ -UTP (10-30 Ci/mmol).

Preparation of Oocyte, Egg and Embryo Protein Extracts.

Washing and Homogenizing Buffer (W-H Buffer).

50 mM Tris-HCl pH 8.0, 0.5 mM DTT, 0.05 mM EDTA.

Eggs and embryos were dejellied in 1% cysteine hydrochloride pH 8.0. Small pieces of ovary or dejellied eggs or embryos were washed in several changes of W-H buffer and then homogenized in a half volume of W-H buffer in a Teflon-glass homogenizer on ice. The resultant homogenate was given a clearing spin (MSE 6L, 8 x 50 ml rotor, at 2500 rpm for 30 minutes at 4°C) before the supernatant was centrifuged further (MSE HS18, 8 x 5 ml rotor, at 12600 rpm for 30 minutes at 4°C). The extracts were collected free from lipid, yolk and pigment and stored at -70°C in 100 μ l aliquots. Alternatively, if the original crude extract volume was small, the homogenate was given a single spin in an Eppendorf microfuge (3 minutes at 4°C). Protein content was determined using the method of Lowry et al (1951) and adjusted to 30 mg/ml.

Protein extracts centrifuged at 100,000g were spun in a 3x6.5ml rotor, MSE 6L, 3 hrs at 4°C before being stored as above.

Preparation of ^{35}S -oocyte Extract.

About 200 vitellogenic oocytes were incubated in 250 μl modified Barth-X with 200 $\mu\text{Ci}^{35}\text{S}$ -methionine for 24 hours. After this time the oocytes were washed thoroughly in WH buffer and a soluble protein extract made as described above.

Column Chromatography.

Column Buffer

10 mM Tris-HCl pH 8.0, 0.05 mM EDTA, 0.5 mM DTT.

Sephadex G100 was swollen in column buffer overnight and de-fined. A column (height 23 cm, diameter 2 cm) was packed and washed through with column buffer at 4°C. The column was calibrated with cytochrome c, ovalbumin and blue dextran as markers. 50 μl glycerol was added to the 200 μl protein extracts before loading on the column. The resulting eluate was collected as 30 drop fractions.

Preswollen DEAE cellulose was packed (column size; height 2.5 cm, diameter 2 cm) and washed with column buffer. 200 μl samples of crude protein extracts or 3 ml volumes of G100 column fractions were loaded and further column buffer run through until the OD_{280} of the eluate was zero. A NaCl gradient was run through the washed column (0-0.45 M NaCl in column buffer) and 30 drop fractions collected.

All columns were run at 4°C, and the resulting column fractions stored at -20°C.

Acrylamide Gel Electrophoresis.

Running Buffer

50 mM Tris-HCl pH 8.0, 380 mM glycine, 0.1 % SDS.

Sample Buffer

50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β -mercaptoethanol, 0.001% BPB.

18% low bis acrylamide gels were run (18% acrylamide, .09% bis-acrylamide, 37 mM Tris-HCl pH 8.8, 0.5% SDS), with a 3% acrylamide stacking gel (3% acrylamide, 24 mM Tris-HCl pH 6.8, 0.1% SDS). The resulting gels were either stained with Coomassie Blue or Silver stained (Switzer et al, 1979)

Coomassie Blue Staining.

Stain and Fix

45% ethanol, 10% glacial acetic acid, 0.1% coomassie blue.

Destain I.

45% ethanol, 10% glacial acetic acid.

Destain II.

5% isopropanol, 10% glacial acetic acid, 0.005% Coomassie blue.

Gels were stained for a minimum of 3 hours, followed by 2 hours in each destain.

Silver staining.

The original method of Switzer et al (1979) was used.

Fluorography

The fixed gels were treated with DMSO (two washes) and then DMSO plus PPO, before being dried down and exposed to X-ray film (Fuji RX).

Extraction of RNA

a. From Whole Cells.

Modified Kirby (1968) Extraction Buffer.

10 mM EDTA, 6% 4 amino salicylic acid, 1% NaCl, 0.1 M Tris-HCl pH 9.0, 2% SDS, 6% phenol.

The cells (eg. tissue culture cells, Xenopus laevis oocytes) were homogenized in an equal volume of extraction buffer, and extracted with two volumes of phenol-chloroform (1:1) until the interface was clear. The aqueous phase was extracted twice more with chloroform-isoamylalcohol (1:24) made 0.4 M LiCl and precipitated with 2.5 volumes of ethanol at -20°C overnight. The precipitated nuclei acids were redissolved in 5 mM MgCl₂, 10 mM Tris-HCl pH 7.5 and incubated on ice with 100 µg/ml DNase I for 10 minutes. This was phenol extracted and precipitated after the DNase treatment. The resulting RNA precipitate was reprecipitated at least once more before being stored at -20°C as the precipitate until required.

b. From Transcription Assays.

Modified Kirby (1968) Extraction Buffer.

1% tri-isopropyl-naphthalene, 5% phenol, 6% 4-amino-salicylic acid, 50 mM Tris-HCl pH 8.2.

An equal volume of 2 x extraction buffer was mixed well with the transcription assay. The procedure for extraction was the same as for a whole cell RNA extraction, except that the volumes were much smaller (about 1 ml) and therefore the extractions were carried out in Eppendorf Fliptop 1.5 ml vials and using an Eppendorf benchtop microfuge.

Preparation of Plasmid DNAs.

The bacteria were grown in L-Broth until they reached on OD₆₅₀ of 0.8 to 1.0. At this point chloramphenicol was added to 50 µg/ml and the bacteria kept at 37°C for 20 hours. The bacteria were pelleted (MSE HS18, 6x250 ml rotor, 15 minutes at 4°C) and taken up in 25% sucrose, 50 mM Tris-HCl pH 8.0. The bacteria were lysed with 5 mg/ml Lysozyme on ice for 5 minutes, followed by the addition of 0.2 volumes of 0.25 mM EDTA, 50 mM Tris-HCl pH 8.0 and 2 volumes 0.25 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.5% Triton-X100 for 20 minutes. After lysis the bacterial mix was spun at 18,000 rpm in the MSE HS18 centrifuge (8x50 ml rotor) and the supernatant stored at 4°C.

The plasmid DNA was purified from the cleared lysate by the method of Colman et al (1978) and stored as precipitated DNA at -20°C until required.

Loading DNA onto Nitrocellulose Filters.

DNA in 1xSSC was denatured by incubating the DNA in 0.5 M NaOH for 45 minutes at room temperature. The Millipore filters (Type HA, pore size 0.45 μ m) were washed with distilled water and 6xSSC before the denatured DNA was neutralized with HCl and loaded onto the filter under slight negative pressure. The filters were washed once more with 6xSSC before being baked at 80°C for 2 hours and stored at -20°C.

RNA-DNA Hybridizations.

The Millipore filters loaded with the appropriate DNA were dampened with 3xSSC, 50% formamide and carefully placed at the bottom of a plastic scintillation vial insert. The RNA sample to be hybridized was taken up in 3xSSC, 50% formamide pH 7.0 and added to the filters. These were incubated submerged in a 37°C water bath for 24 hours. The hybridized filters were pooled in a large volume of 0.2% SDS, 2xSSC and shaken gently while being washed (4x30 minute washes). The dried filters were counted in a Packard Scintillation Counter with PPO-POPOP-Toluene scintillant.

Agarose slab gel electrophoresis.

Tris-Acetate Running Buffer.

40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, adjusted to pH 8.3 with glacial acetic acid.

Sample Buffer.

0.5 x Running Buffer, 1% SDS, 25% glycerol, 2% BPB.

Standardly 0.8% agarose gels were poured into a 15 cm x 20 cm mould with slot former and used as soon as it had set. The gels were run submerged at 25 v overnight or until the marker dye had migrated two thirds of the way down the gel. Gels were stained in 50 µg/ml ethidium bromide for 15 to 30 minutes, before being photographed under UV light. Gels that were to be Southern blotted were not stained or examined under UV light, instead the marker tracks were cut away and these were stained whilst the rest of the gel was blotted.

Transfer of DNA Fragments from Agarose Gels to Nitrocellulose Filters.

This was carried out using the procedure of Southern (1975); the agarose gels were soaked in 0.5 M NaOH, 1.5 M NaCl for 15 minutes followed by 1 M Tris-HCl pH 5.0, 3 M NaCl for a further 15 minutes. The treated gel was placed on dampened filter paper over a reservoir of 20xSSC with a nitrocellulose filter on top of the gel. Further dry filters and tissues were placed above this. The transfer was allowed to run for 24 hours before the filter was removed, washed in 2xSSC and baked at 80°C for 2 hours.

Separation of RNA Species using Sucrose Gradients.

Sucrose solutions (20% and 5%) and the RNA (at a maximum of 100 ug per gradient) were in 50 mM Tris HCl pH 7.5, 100 mM NaCl, 0.5 mM EDTA. All solution used were DEPC treated and the sucrose was RNase-free grade. 12 ml linear 5-20 % sucrose gradients were made and loaded with 100-200 µl RNA sample. These were centrifuged in a Beckman L8 centrifuge (SW40 rotor, 38,000 rpm, 4°C, 4 hours) and fractionated into 0.4 ml fractions using a density gradient fractionator (ISCO, model 640).

DNA Extraction.

Extracted nuclei were taken up in 0.5% SDS, 12.5 mM EDTA and incubated overnight with 50 mg/ml proteinase K. The resulting mixture was extracted with phenol-chloroform (1:1, equilibrated against 10 mM Tris-HCl pH 7.5, 1 mM EDTA) until the interface was clear, and twice with chloroform-isoamylalcohol (24:1). The aqueous phase was made 0.2 M sodium acetate and precipitated with 2.5 volumes of ethanol.

The precipitate was redissolved in 0.1xSSC, made 0.3 M sodium acetate and incubated for 60 minutes at 37°C with 100 µg/ml RNase A and 1,000 units T1 RNase. This was extracted with phenol-chloroform twice and chloroform-isoamylalcohol once before being precipitated with 2.5 volumes of ethanol.

The DNA was redissolved in 10 mM Tris-HCl pH 8.5, 1 mM EDTA and dialysed against this buffer for 24 hours, before being reprecipitated, redissolved in water or the dialysis buffer and the concentration calculated. The DNA was stored at 4°C for immediate use or as an ethanol precipitate at -20°C.

DNase I Digestion.

RSB.

0.01 M Tris-HCl pH 7.4, 0.01 M NaCl, 3 mM MgCl₂.

Proteinase Buffer.

10 mM EDTA, 0.1% SDS.

DNase I Stopping Solution.

6.7% SDS, 167 mM EDTA

The method of Weintraub and Groudine (1976) was used. Erythrocyte nuclei were gently centrifuged out of the preincubation mix, or if this step had not been carried out, from RB, and taken up in RSB such that the final concentration was 1 mg/ml DNA (1.67×10^8 nuclei/ml). DNase I at 1 mg/ml was added to a final concentration of 20 µg/ml and the nuclei incubated at 37°C for 5 minutes when 5-10% of the DNA had been released. The nuclei were gently spun out of the digestion buffer and resuspended in twice the volume of proteinase buffer. Proteinase K was added and the DNA extracted as outlined above.

In order to investigate whether there were any sites of high DNase I sensitivity (Wu et al., 1979a) present, the nuclei were DNased for a shorter time and the reaction was stopped by making the reaction 12.5 mM EDTA; 0.5% SDS with the stopping solution. The DNA was extracted in the normal way.

DNA-DNA Hybridization Conditions.

Hybridization buffer.

50% Formamide pH 7.0, 3xSSC, 0.2% BSA, Ficoll, PVP, 0.25 mg/ml non-competing yeast RNA.

1. Dot Blots.

Nitrocellulose filters loaded with DNA were dampened with the hybridization buffer and placed in the bottom of a scintillation vial insert or bijou. Hybridization buffer was added (a final volume of 100 μ l buffer + probe per two duplicate filters) and the denatured nicktranslated DNA probe in 0.1xSSC. The vials were capped and hybridized at 37°C for 24 hours. The filters were washed after the hybridization in three washes (30 minutes each) of 2xSSC at room temperature.

2. Southern Blots.

The same basic method as that described above was used, but scaled up such that 5 ml buffer + probe was used for each filter. These were hybridized within a sealed plastic bag for 24 hours before being washed as above.

Nicktranslation of DNA

Nicktranslation Buffer.

50 mM Tris-HCl pH 7.9, 10 mM MgCl₂, 10 mM DTT, 50 mg/ml BSA.

For every γ pmoles hot dNTP 2 γ pmoles cold dNTPs were used, usually 50 μ Ci labelled dNTP was used, either ³H-dCTP (18-25 Ci/mmol) or ³²P-dCTP (2000-3000 Ci/mmol). These were frozen in liquid nitrogen and desiccated immediately before being taken up in nicktranslation buffer. DNA (from 100 ng to 1 μ g per reaction) at a final concentration of 1 μ g/100 μ l and DNase I were added such that for every μ g DNA 100 pg DNase I was used, and incubated for 15 minutes at 37°C. 5 units Kornberg polymerase I were added and the reaction incubated for 3 hours at 15°C. Carrier DNA was added to the reaction which was phenol extracted and desalted by running through a Sephadex G75 column. The fractions were counted and the first peak precipitated. The resulting probe was redissolved in 0.1xSSC, and denatured by boiling and rapid cooling before being added to the hybridizations.

CHAPTER III; TRANSCRIPTION IN ERYTHROCYTE NUCLEI AND THE EFFECT OF CELLULAR EXTRACTS.

A faithful transcription system consists of the template, enzyme, triphosphates and also the salts necessary to obtain optimal incorporation of the triphosphates into the RNA product. A variety of templates have been used, extending from recombinant and total nuclear DNA to chromatin and intact nuclei. These have been transcribed by a range of added enzyme preparations; purified and semipurified RNA polymerases from the same source as the template or from E.coli, cellular extracts and the endogenous RNA polymerases that are in the nuclei or chromatin being assayed. Optimal activity is measured not only by overall incorporation of radioactivity, but also by assessing the amount of faithful transcription.

Whole nuclei have been used to study a number of genes, such as those encoding the Drosophila heat shock protein genes (Miller and Elgin, 1981) and mouse immunoglobulin kappa light chain genes (Smith and Huang, 1976). In several cases pure, cloned genes have been used as templates eg Xenopus 5S rRNA (Engelke et al, 1980), mouse rRNA (Grummt, 1981) and chicken conalbumin and ovalbumin (Wasylyk et al, 1980). Most of these types of assay system utilize homologous RNA polymerases, but whilst some success has been claimed using chromatin plus E.coli RNA polymerase to synthesize rabbit globin mRNA (Wilson et al, 1975), other workers (Zaslhoff and Felsenfeld, 1977; Maryanka et al, 1979) have been unable to produce avian globin mRNA using the same sort of system.

While it is clearly important to have an homologous template

and RNA polymerases, it is also important to have the correct salt concentrations to give the optimal transcription conditions. Most transcription systems have monovalent cations (either K^+ or NH_4^+) at a fairly high concentration (50-150 mM). These correspond to an extent on the normal isotonic environment of the nucleus in the cell, for example in Amphibia it is 110 mM. The necessary divalent cations (Mg^{2+} and/or Mn^{2+}) are present at much lower concentrations (0-10 mM), (Marzluff et al., 1973; Crampton and Woodland, 1979a; Thomas et al., 1980; Miller and Elgin, 1981). The activity of all three of the RNA polymerases found in Xenopus laevis depend on the concentration of magnesium or manganese ions, and all have slightly differing salt optima (Roeder, 1974a). Whilst all will function with 1-2 mM Mn^{2+} , RNA polymerases II and III require less Mg^{2+} (4 mM and 2 mM respectively) than RNA polymerase I (6 mM). Some systems (Miller and Elgin, 1981) function without manganese and contain only magnesium as the divalent cation. Others (Crampton and Woodland, 1979a) will function entirely on manganese.

There is also a balance to be maintained between a high radioactive incorporation, and a reasonable absolute level of synthesis for initiation and elongation by the RNA polymerases. This will depend on various factors, such as the actual activity of the RNA polymerases on the particular template.

Preparation of Erythrocyte Nuclei.

The blood collected from one adult female Xenopus laevis (5-7 ml/animal) was diluted into calcium-magnesium free modified Barth's medium and washed free of contaminating white blood cells by gentle centrifugation and resuspension in the buffer at 4°C.



1. The first part of the experiment was to determine the effect of the concentration of the solution on the rate of reaction.
2. The second part of the experiment was to determine the effect of the temperature on the rate of reaction.
3. The third part of the experiment was to determine the effect of the surface area of the reactants on the rate of reaction.

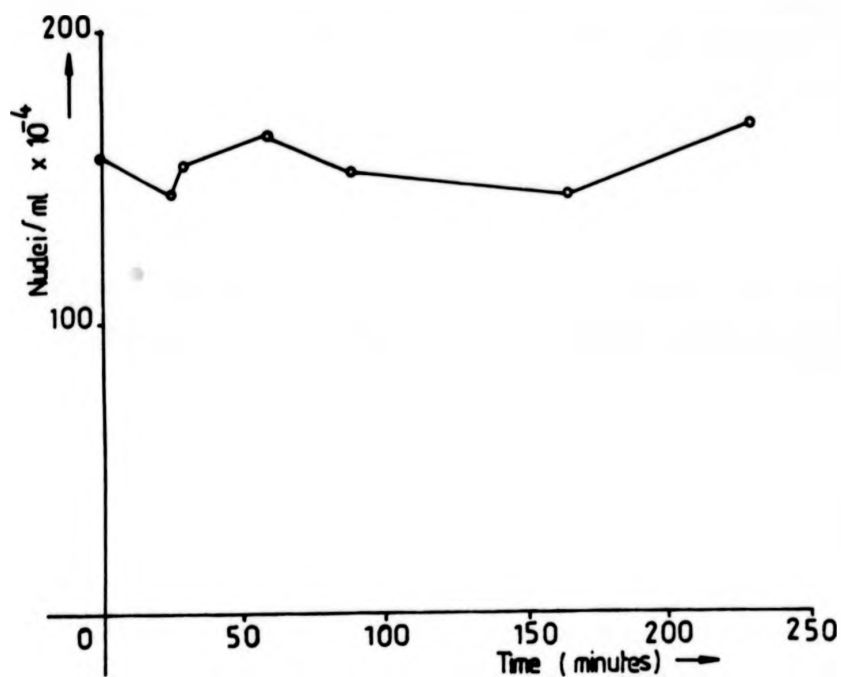


FIGURE 3.1

Nuclear Lysis During Incubation.

Erythrocyte nuclei were incubated in a standard incubation mix at 10^6 nuclei/125 μ l. At times during the incubation 5 μ l samples were removed, diluted into 20 μ l 0.5 x RB and counted in a haemocytometer.

TABLE 3.1

Transcriptional Activity of Stored Erythrocyte Nuclei.

Age of nuclei	pmoles UMP incorp.
	/10 ⁶ nuclei/30 minutes
0 days	5.41
2 days	5.63
2 weeks	5.27
1 month	5.49
3 months	5.50
6 months	5.39

Nuclei, at various times after preparation, were assayed in a standard 50 μ l transcription assay. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the incubation.

The cleaned erythrocytes were lysed on ice by adding H-buffer, which contains both calcium and magnesium as well as 0.1% Triton X100, followed by homogenization in a loose fitting Dounce homogenizer. The nuclear suspension, checked for 100% lysis by phase microscopy, was made up to 1 M sucrose, layered over a 2 M sucrose pad and centrifuged at 4000g for one hour. This gives a soft clean pellet of nuclei. These were resuspended in RB and stored at -70°C for up to 6 months without significant loss in activity [TABLE 3.1].

As compared with other methods (eg Hilder and Maclean, 1974), where nuclei are repeatedly spun for a short time at high speed to remove any contaminating cytoplasm, this method (modified from that of Marzluff et al, 1973 and Crampton and Woodland, 1979a) has the advantage that the nuclei are only spun and resuspended once, so minimising any loss of transcriptional activity. The nuclei are rendered free from cytoplasmic contamination by passage through the sucrose pad. Nuclei isolated using Triton X100 tended to clump, so all incubates were gently agitated during the assay to prevent this clumping and to ensure even distribution of the assay components. Loss by nuclear lysis was undetectable under these conditions, even after 2 hours of incubation [FIG 3.1].

Salt Optima for Erythrocyte Nuclear Transcription.

As a starting point, the conditions of Marzluff et al (1973) were used, but with a lower UTP concentration (0.02 mM, instead of 0.05 mM UTP; in addition 4 $\mu\text{Ci}^3\text{H}$ -UTP, at 10-30 Ci/mole, were present in 100 μl assays). It was decided to optimize the nuclei for maximal incorporation of ^3H -UTP into TCA

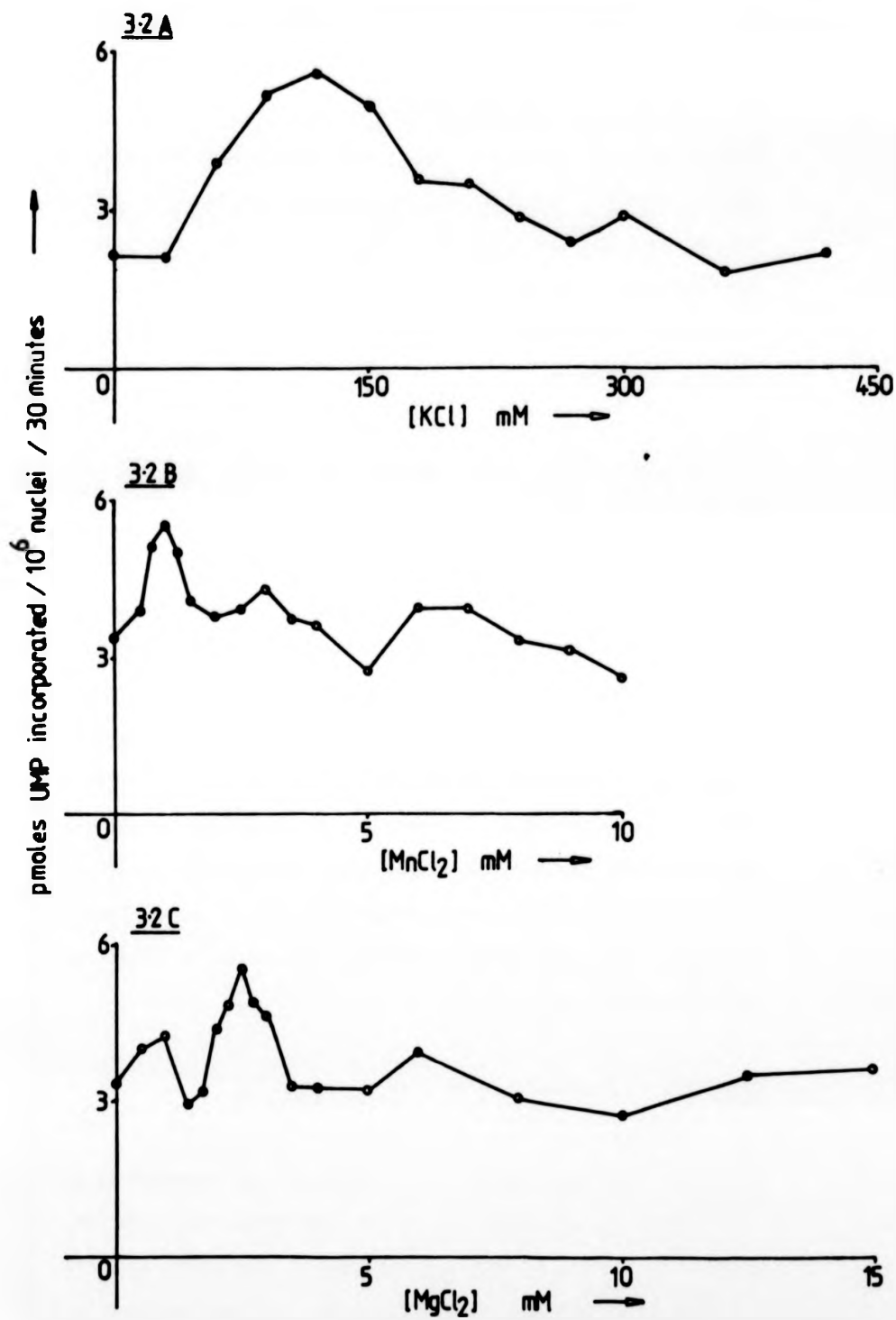


FIGURE 3.2

Salt Optima.

Throughout this series of assays the concentrations of ATP, GTP, and CTP were kept constant at 0.4 mM. The concentration of cold UTP was 0.02 mM with 2 $\mu\text{Ci } ^3\text{H-UTP/50 } \mu\text{l}$ assay.

3.2A; KCl Optima. A series of 50 μl assays, containing 5×10^5 nuclei in 0.5 x RB with 2.5 mM MgCl and 1 mM MnCl, were set up with a range of KCl concentrations between 0 and 420 mM. These were incubated at 25°C. Duplicate 10 μl samples were taken at 0 and 30 minutes.

3.2B; MnCl Optima. A series of 50 μl assays, containing 5×10^5 nuclei in 0.5xRB with 2.5 mM MgCl and 120 mM KCl, were set up with a range of MnCl concentrations between 0 and 10 mM. These were incubated at 25°C. Duplicate 10 μl samples were taken at 0 and 30 minutes.

3.2C; MgCl Optima. A series of 50 μl assays, containing 5×10^5 nuclei in MgCl free 0.5xRB, 120 mM KCl and 1 mM MnCl, were set up with a range of MgCl concentrations between 0 and 15 mM. These were incubated at 25°C. Duplicate 10 μl samples were taken at 0 and 30 minutes.

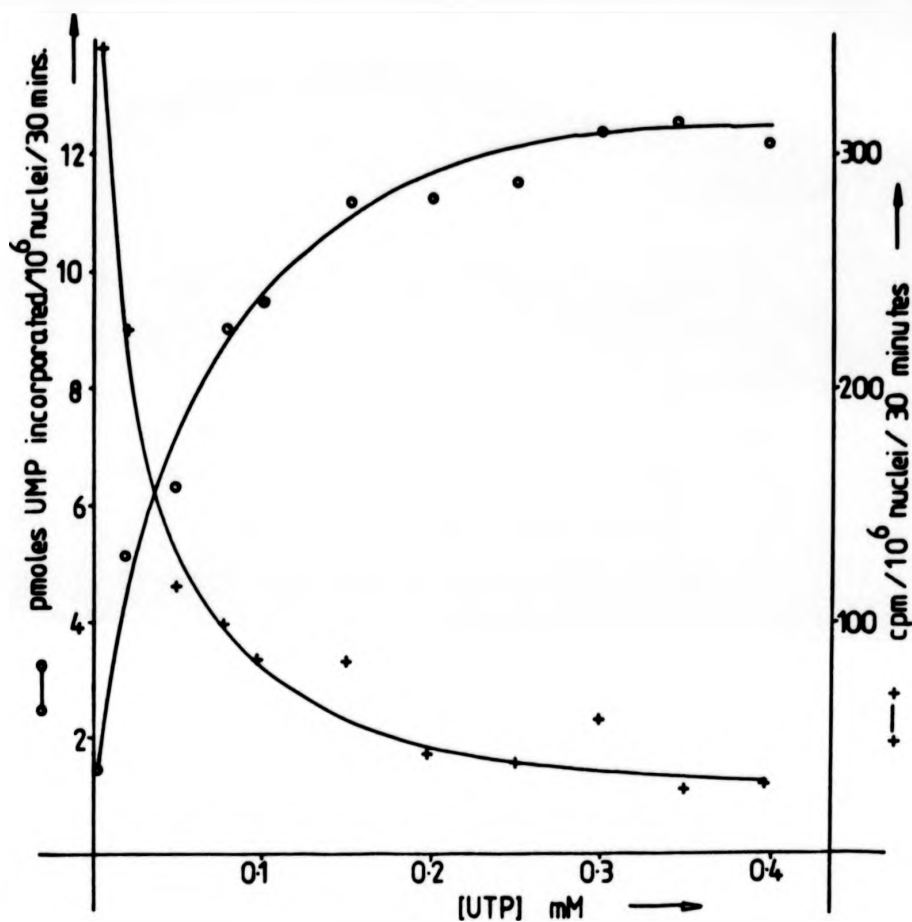


FIGURE 3.3

Specific Activity of ³H-UTP in the Transcription Assay.

A series of 50 μ l assays were set up (5×10^5 nuclei, 0.5xRB, 120 mM KCl, 1 mM MnCl, 0.4 mM ATP, CTP, GTP, and 2 μ Ci ³H-UTP) with varying amounts of cold UTP from 0 to 0.4 mM. Duplicate 10 μ l samples were taken at 0 and 30 minutes.

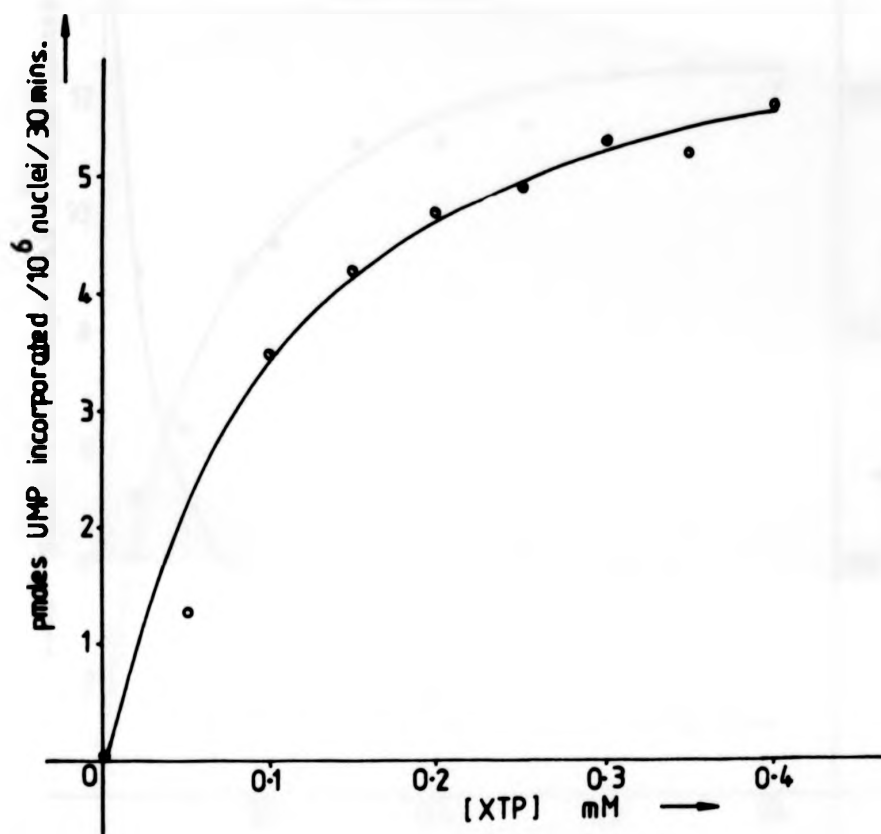


FIGURE 3.4

Triphosphate Optimum.

A series of standard 50 μ l assays were set up but without ATP, CTP and GTP. These were added in varying concentrations between 0 and 0.4 mM. Duplicate 10 μ l samples were taken at 0 and 30 minutes.

precipitable counts without added cell extracts. All incubations were carried out at 25°C.

To determine which concentration of KCl gave maximum incorporation of ^3H -UTP into TCA insoluble material, the Mn^{2+} and Mg^{2+} concentrations were kept constant at 1.0 mM and 2.5 mM respectively whilst the K^+ concentration was varied from 0 mM to 420 mM. Figure 3.2A shows that the amount of incorporated was maximal at 120 mM KCl. This value was used in all subsequent assays. The optimum concentration of MnCl is 1 mM [FIG 3.2B], and that of Mg^{2+} was demonstrated to be 2.5 mM [FIG 3.2C].

The optimal levels of radioactive and non-radioactive nucleotide triphosphate concentrations were also determined. Firstly the amount of non-radioactive UTP was varied. The other three triphosphates were kept at 0.4 mM and the ^3H -UTP label at 2 $\mu\text{Ci}/50 \mu\text{l}$ assay. Secondly, the concentration of ATP, CTP and GTP was varied while both cold and labelled UTP were kept constant.

At very low concentrations of cold UTP (less than 0.005 mM) although the radioactivity incorporated is high, the actual number of pmoles of UMP incorporated into RNA is very low [FIG 3.3]. At the other extreme, 0.4 mM cold UTP, the opposite is true, and the resulting transcription by the erythrocyte nuclei cannot be accurately measured. A concentration of 0.02 mM was finally used in subsequent assays. Although this does not give maximum elongation and initiation by the RNA polymerase molecules, or the largest amount of radioactivity incorporated, it is a balance between the two. Figure 3.4 shows that there is no pool of triphosphates in the erythrocyte nuclei - adding no ATP, CTP and GTP results in no incorporation of label. After 0.3

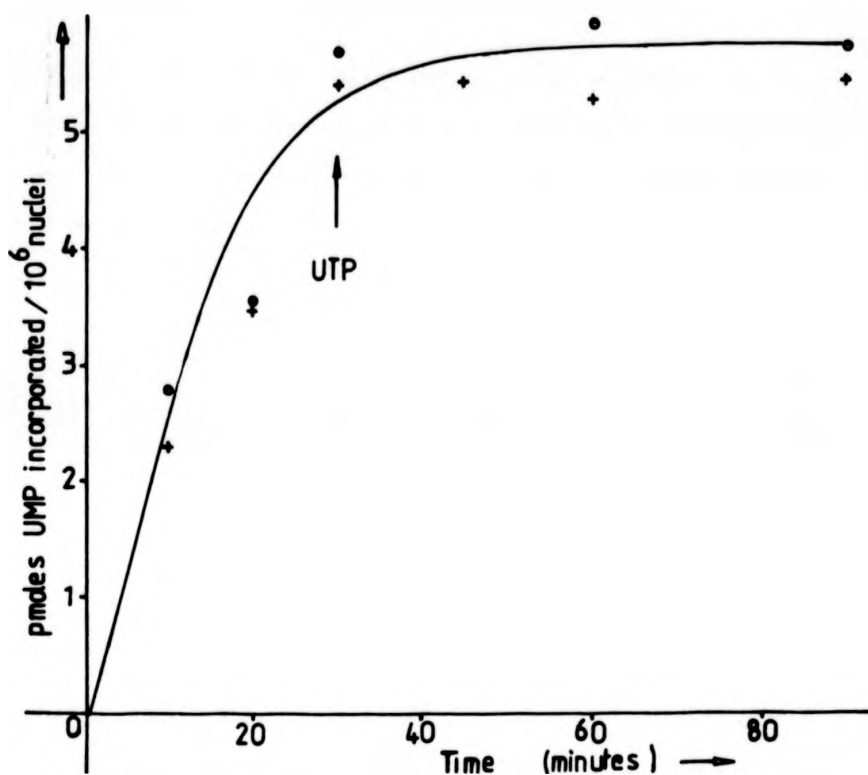


FIGURE 3.5

Test for Degradation of RNA Product of Transcription by Erythrocyte Nuclei.

Two duplicate 200 μ l assays (2×10^6 nuclei) were incubated at 25°C. At intervals two 10 μ l samples were taken to determine the pmoles UMP incorporated/ 10^6 nuclei at that time point. One assay (o---o) was left unchanged throughout the 90 minute incubation, The other (+---+) had unlabelled UTP added after 30 minutes to a final concentration of 0.5 mM.

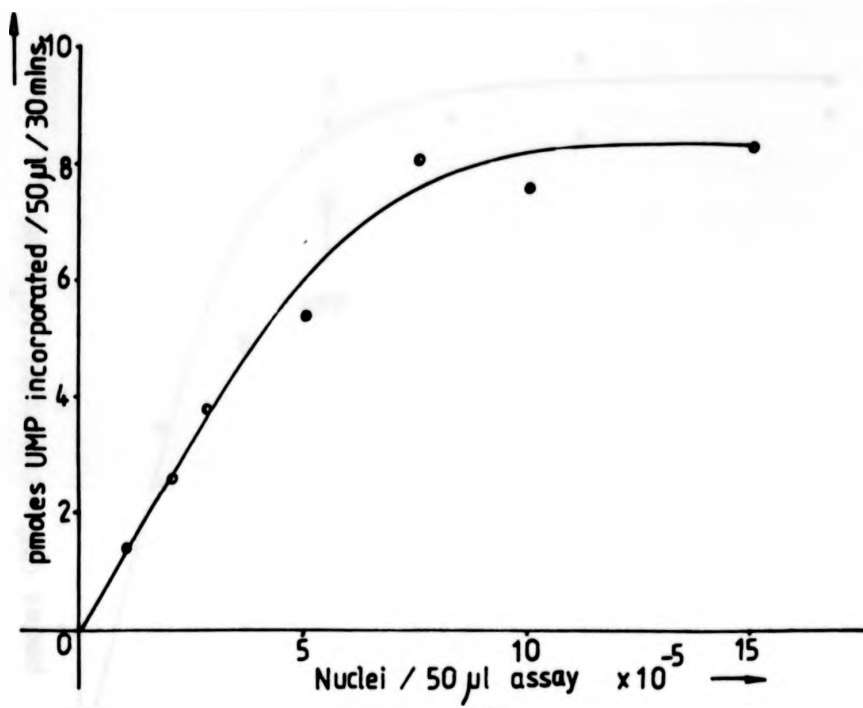


FIGURE 3.6

Number of Erythrocyte Nuclei in the Transcription Assay.

The number of erythrocyte nuclei in a standard 50 μ l transcription assay was varied from 0 to 15×10^5 . Duplicate 10 μ l samples were taken at 0 and 30 minutes.

mM XTP, no further increase in incorporation is seen, this agrees well with the UTP concentration curve [FIG 3.3]. Therefore a concentration of 0.4 mM was used for the unlabelled triphosphates.

RNA Polymerase Activity in Erythrocyte Nuclei.

If the transcription by erythrocyte nuclei in the system described above is followed over 90 minutes [FIG 3.5], it can be seen that the incorporation of UMP into acid-insoluble material is linear for 30 minutes. After this time the reaction slows and gradually plateaus off, such that the maximal amount of UMP incorporated is about 5.5 pmoles; that is one erythrocyte nucleus synthesizes about 0.006 pg RNA in 30 minutes. The level of synthesis can therefore be calculated as 0.1% of the DNA content.

If cold UTP is added to a final concentration of 0.5 mM - an amount sufficient to arrest detectable incorporation- at 60 minutes of the incubation, no decrease in the amount of ^3H -UTP incorporated can be detected, as would have been the case if RNase had been present [FIG 3.5].

That the transcription observed is dependent upon the numbers of nuclei present in the assay is shown in Figure 3.6. The correspondence between nuclear numbers and the amount of ^3H -UTP incorporated is linear up to 7.5×10^5 nuclei/50 μl assay. At this value, the graph plateaus off. Also, at higher concentrations of nuclei, they become more likely to clump and perhaps for this reason the distribution of the assay components is more uneven, causing the levelling off. An assay with no nuclei present incorporates no radioactive precursor. In a

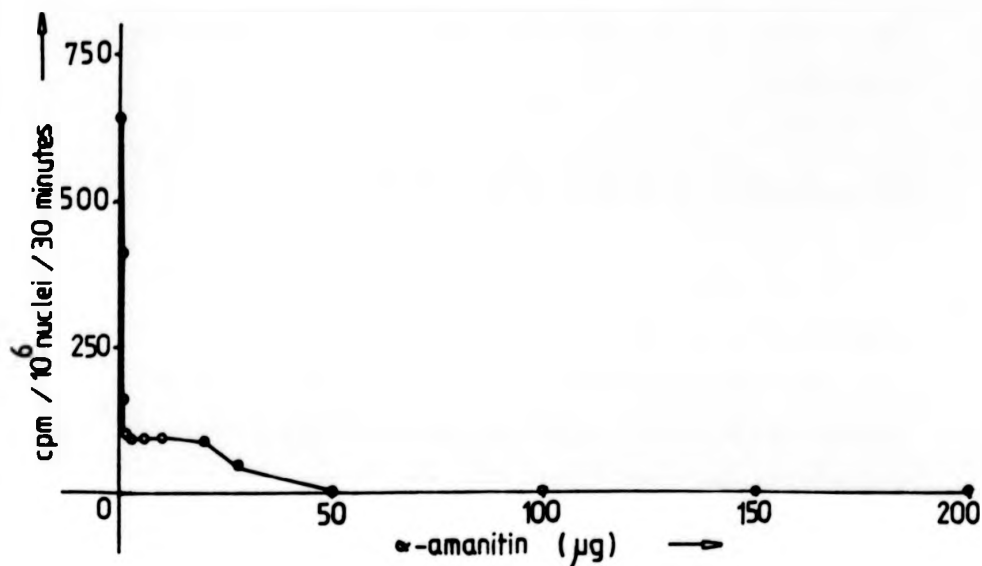


FIGURE 3.7

Inhibition of Transcription in Erythrocyte Nuclei by α -amanitin.

A series of 50 μl standard transcription assays were set up with increasing amounts of the inhibitor α -amanitin. Duplicate 10 μl samples were taken at 0 and 30 minutes and incorporation was expressed as TCA-insoluble cpm/ 10^6 nuclei.

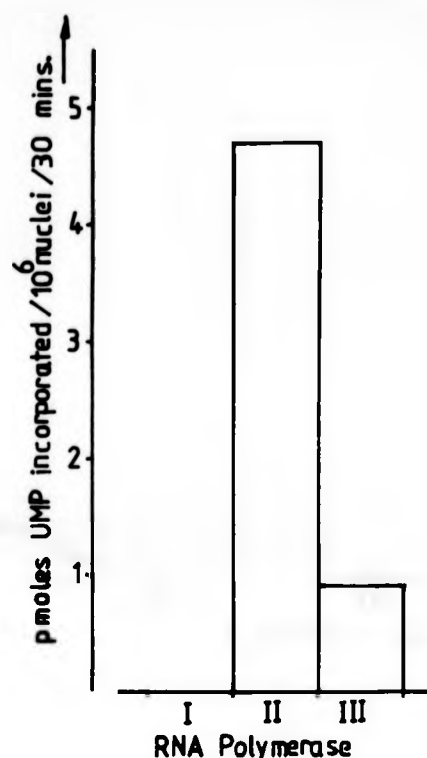


FIGURE 3.8

Relative Levels of RNA Polymerases I, II, and III in Erythrocyte Nuclei.

To duplicate 50 μ l transcription assays was added α -amanitin, at either 1 or 100 μ g/ml. Assays with no α -amanitin were also carried out. 10 μ l samples were taken at 0 and 30 minutes and the pmoles UMP incorporated/ 10^6 nuclei calculated for each α -amanitin concentration. This was expressed as the amount of transcription due to each of the three RNA polymerases.

standard assay of 50 μ l there were 5×10^5 nuclei present (10^7 nuclei/ml), which are enough to give a readily measurable incorporation without problems of clumping.

In order to investigate the relative activities of the three RNA polymerases in the erythrocyte nuclei, use was made of the inhibitor α -amanitin (Jacob et al, 1970). At low concentrations (1 μ g/ml) α -amanitin specifically inhibits RNA polymerase II, which synthesizes hnRNA and mRNA. At higher concentrations (100 μ g/ml) RNA polymerase III, responsible for 5S and tRNA synthesis, is inhibited. The synthesis of rRNA by RNA polymerase I is not inhibited by α -amanitin. If the concentration of α -amanitin is increased in the nuclear assay [FIG 3.7] it can be seen that the low concentrations of α -amanitin (0.1-20 μ g/ml) decrease the transcriptional activity to very low levels. On increasing the concentration further (above 50 μ g/ml) all detectable transcription is inhibited. Thus, the relative activities of the three RNA polymerases can be calculated [FIG 3.8].

Almost all the transcriptional activity seen is due to RNA polymerase II activity (86%). There is some RNA polymerase III activity (14%), but no α -amanitin resistant RNA polymerase I activity is detectable. These values agree well with the data of Hentschell and Tata (1978) who showed that the RNA polymerase activity seen in mature erythrocyte nuclei is due to the persisting, template-bound, inactive RNA polymerase II molecules, although they found a low level (3%) of 1 μ g/ml α -amanitin resistant RNA polymerase activity.

During the development of the mature erythrocyte from the

basophilic erythroblast, at each successive division the cell volume gets progressively larger whilst the nucleus becomes more condensed. By the time the erythrocyte is mature its nucleus/cytoplasm ratio has dropped from 0.8 for the basophilic erythroblast to 0.35 (Thomas and Maclean, 1975). Whilst this is taking place there is a gradual cessation of RNA synthesis. Cells early in erythropoiesis are transcriptionally active, but as they undergo differentiation to the mature form, RNA polymerase I and then RNA polymerase III activity is lost (Hentschell and Tata, 1978). This is due to these enzyme molecules being lost from the cell. In the mature erythrocyte there is still some transcription, but this is due to the remaining RNA polymerase II molecules. These are locked onto the chromatin and the activity seen represents a slow elongation of the final transcript.

In avian erythrocytes, which undergo a similar development to that found in Xenopus (Williams, 1972), it has been found that RNA polymerase II molecules are tightly bound to the 5' ends of the globin genes (Gariglio et al, 1981). It would seem likely therefore, that the RNA polymerase II molecules in Xenopus erythrocytes are also to be found in similar transcriptional complexes on the globin genes. Thus the erythrocyte nuclei prepared and assayed by the methods discussed above would appear to conform to previously published data on erythrocyte transcription (Hentschell and Tata, 1978). Most of the synthesis seen is RNA polymerase II activity, whilst the remainder, calculated from a figure not much above background, is due to RNA polymerase III transcription. This could be because of some late erythrochromatic erythroblasts are present in the blood sample; the lack of any RNA polymerase I activity would argue against any contamination by very early stage erythroblasts or

white blood cells.

Effect of Oocyte and Egg Extracts on Erythrocyte Transcription.

As previously discussed, the transition from oocyte to egg brings about an abrupt reduction in transcriptional activity. The high level seen during oogenesis falls and no measurable rRNA synthesis is seen until the mid to late blastula stage of development (Shiohawa et al., 1981a, b). Considering a single gene or gene family, the transition between the different activity states could be seen simplistically as resulting from an inhibitor in the egg which is later inactivated or diluted out by the subsequent cell divisions of the developing embryo. Alternatively, there might be an activator in the oocyte which is lost or chemically modified in some way during the egg stage and reappears in the later stages of early development. The existence of a definite activating molecule has been shown in the case of the oogenetic 5S genes of Xenopus (Birkenmeier et al., 1978; Ng et al., 1979; Engelke et al., 1980; Honda and Roeder, 1980). This activator is present in oocytes, but is absent from extracts of unfertilized eggs. While these experiments made use of naked recombinant DNA, the erythrocyte provides a good source of equally non-transcribing genes, but in their native chromatin form. This template will detect not only activating molecules which bind to the DNA itself, as in the case of the 5S genes, but also those which interact with the chromatin. However, although the transcriptionally inactive erythrocyte nucleus is a potentially good tool for the study of activating molecules, the fact that it is so inactive means that it is not useful to detect any inhibitors of transcription.

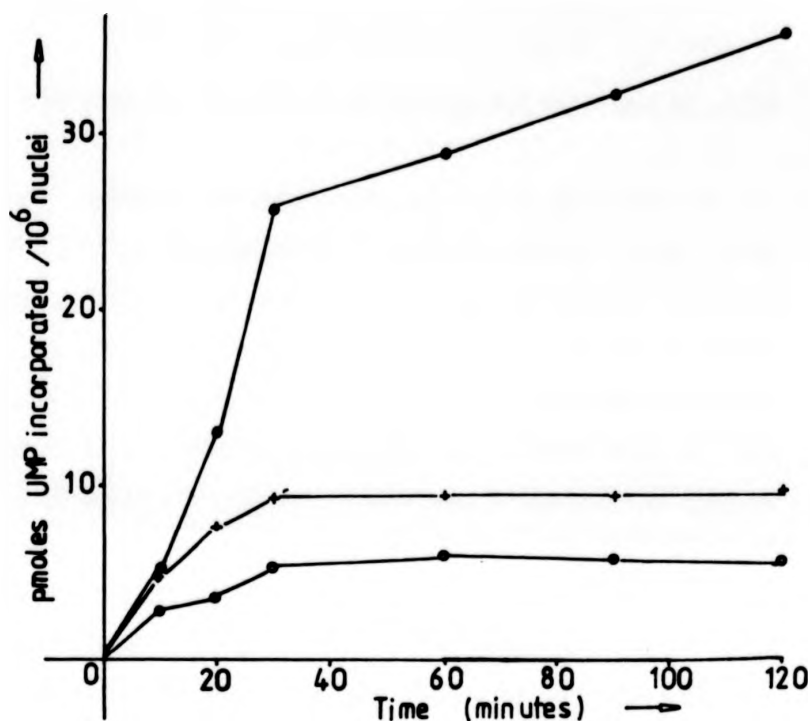


FIGURE 3.9

Effect of Oocyte and Egg Extracts on Transcription by Erythrocyte Nuclei.

To 2×10^6 nuclei was added 37.5 μ l of an oocyte extract (e---e), an egg extract (+---+), the extracts contained 30 mg protein/ml. As a control the nuclei were incubated with 30 mg/ml BSA in column buffer (o---o). Salts and triphosphates were added to give the normal concentrations in a final volume of 200 μ l. Duplicate 10 μ l samples were taken from each vial at intervals throughout the 120 minute incubation.

Extracts of both oocytes and dejellied eggs were prepared by homogenization followed by centrifugation to remove pigment, yolk and lipid from the soluble protein extract. The protein content was determined by the method of Lowry et al (1951) and adjusted to 30 mg/ml. This represents under normal assay conditions 600 µg extract protein/ 50 µl assay or 1.2 ng extract protein/ nucleus. In the case of nuclei treated with an oocyte extract, the soluble protein from 10 oocytes was incubated with 10^6 nuclei.

If an oocyte or egg extract is added to erythrocyte nuclei and the incorporation of ^3H -UTP into acid-insoluble material followed [FIG 3.9], it is found that both extracts increase the synthesis of ^3H -RNA as compared with nuclei that have been incubated with the same amount of protein in the form of BSA, but the oocyte extract increases it to a more marked degree. The addition of the egg extract increases the initial rate of synthesis by about 1.5 times, but the nuclei plateau at about the same time, albeit at a higher level, than the BSA-treated nuclei. The incubation of nuclei with an oocyte extract not only causes the initial rate to increase, but also delays the plateau by at least 60 minutes. Thus, the addition of an egg extract gives a 1.7 fold stimulation and that of an oocyte extract a 4.8 fold stimulation after 30 minutes of incubation. These values are the mean results of several experiments, which showed little variation.

In order to increase the amount of transcription, the nuclei were preincubated at 25°C with low levels of all four triphosphates plus the relevant extract for one hour, before being gently spun down and resuspended in the standard assay

TABLE 3.2

Effect on Transcription in Erythrocyte Nuclei of Preincubation
with Protein Extracts.

Nuclei preincubated with;	pmoles UMP incorp. / 10^6 nuclei/30 minutes	Stimulation
buffer	5.49	1
egg extract	11.5	2.1
oocyte extract	33.1	6.3

5×10^5 erythrocyte nuclei were preincubated with 20 μ l of protein extract in a 50 μ l volume at 25°C for 60 minutes. At the end of this time, the nuclei were gently spun out of the preincubation media and taken up in a standard 50 μ l incubation mix. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the subsequent incubation.

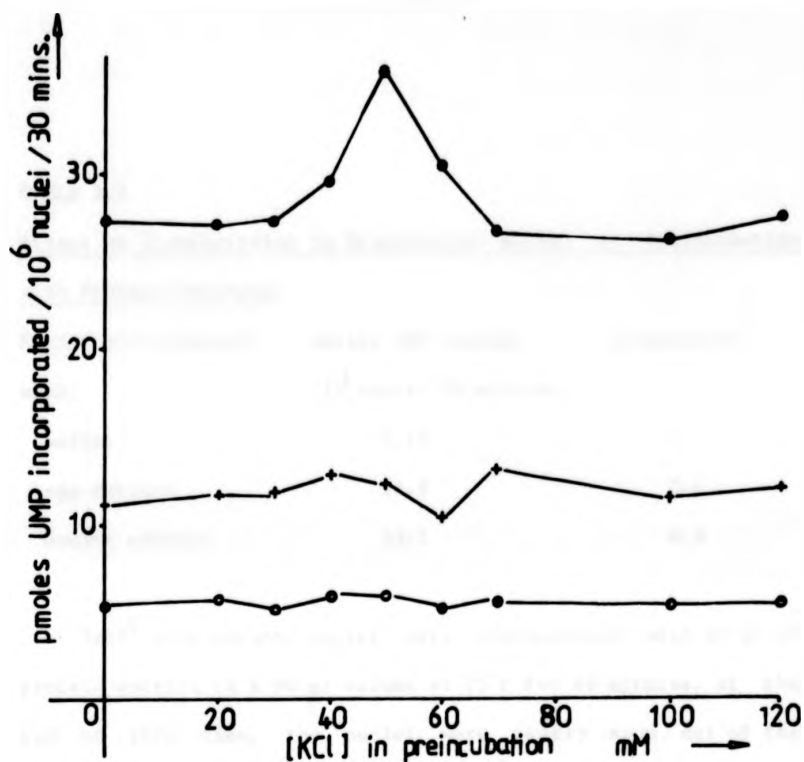


FIGURE 3.10

Optimal Concentration of KCl During Preincubation.

A series of 50 μ l assays (5×10^5 nuclei, 20 μ l protein extract) were set up with either column buffer (o---o), egg extract (+---+) or oocyte extract (●---●) present, and with increasing amounts of KCl from 0 to 120 mM. These were preincubated for 60 minutes, after which time the conditions were adjusted to those of an incubation assay, that is the KCl concentration was brought to 120 mM in all cases. 10 μ l samples were taken at 0 and 30 minutes of the incubation.

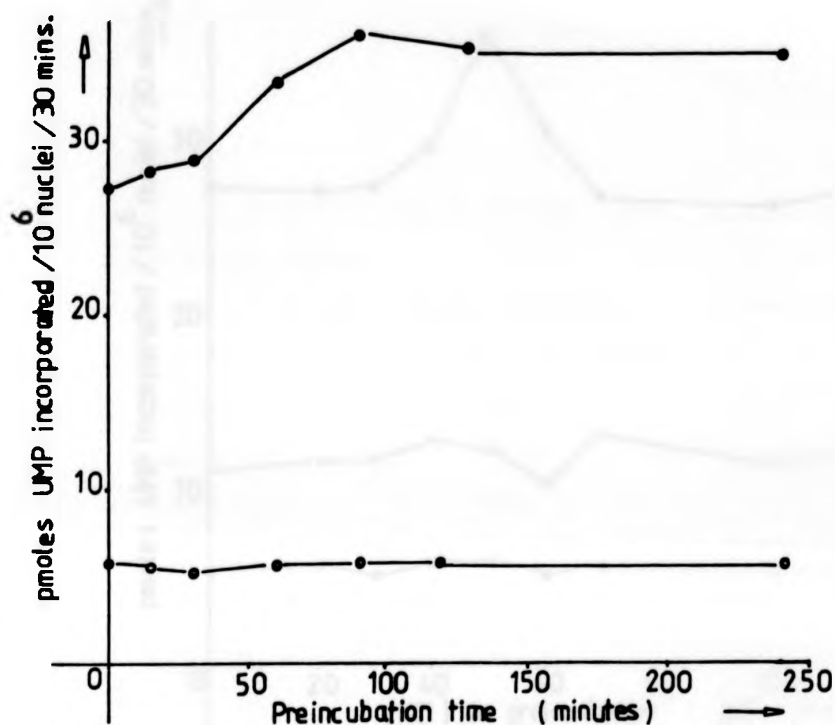


FIGURE 3.11

Preincubation Time.

5×10^5 nuclei in 50 μ l volume were preincubated for increasing lengths of time with either 20 μ l of column buffer (○---○) or oocyte extract (●---●). The nuclei at the end of each preincubation were spun out and resuspended in 50 μ l of incubation mix. Samples (2x10 μ l) were taken at 0 and 30 minutes of the subsequent incubation.

buffer containing ^3H -UTP, and assayed in the normal way. This slightly increases the ratio of extract to nuclei. By preincubating the nuclei in this way, the effect of the egg extract on the nuclei [TABLE 3.2] is increased slightly from a 1.7 fold stimulation to 2.1 fold. That of oocyte extract is also increased to 5.2 fold from 4.8 fold.

Thus whatever agent causes the increases in transcription, must either associate with the nuclei during the preincubation step, and remain with them during centrifugation and resuspension, or its effect must be exerted during preincubation in a stable way. To see if this preincubation effect by the nuclei could be increased by altering the ionic strengths during the preincubation, the KCl concentration was varied during the first stage and brought back to 120mM for the incubation stage. Figure 3.10 shows that at 50mM KCl during the preincubation there is a marked increase in activity in oocyte-treated nuclei, such that a stimulation of 6.0 fold is observed over the control nuclei. This KCl concentration during the preincubation has no detrimental effect on incorporation by the control or egg-treated nuclei, ie this increase in stimulation is because the oocyte-treated nuclei incorporate more ^3H -UTP into TCA-precipitable material, and not because control nuclei synthesize less ^3H -RNA.

The standard preincubation time of 60 minutes is the one that gives the best increase in stimulation by the oocyte extract. A very short, 15 minute, preincubation with oocyte extract will still give a slight increase in incorporation of ^3H -UTP [FIG 3.11], but with a 90 minute preincubation the stimulation is no longer increasing. Further the risk of

TABLE 3.3

Effect on Erythrocyte Nuclear Transcription of Preincubation
with Protein Extracts.

Nuclei preincubated with;	pmoles UMP incorp. /10 ⁶ nuclei/30 minutes	Stimulation
buffer	5.61	1
egg extract	11.8	2.1
oocyte extract	34.2	6.1

5x10⁵ nuclei were incubated with 20 μ l of protein extract in a 50 μ l volume for 60 minutes. After this time, they were supplemented with salts etc to give standard incubation conditions. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the incubation period.

TABLE 3.4

Effect of an Extract of Defolliculated Oocytes on Transcription
by Erythrocyte Nuclei.

Nuclei preincubated with;	pmoles UMP incorp. /10 ⁶ nuclei/30 minutes	Stimulation
buffer	5.61	1
egg extract	11.8	2.1
total ovary extract	34.2	6.1
separated oocyte extract	36.1	6.4

A protein extract was prepared in the standard way from separated and defolliculated oocytes and adjusted to 30 mg protein/ml. 20 μ l of this extract was preincubated with 5×10^5 nuclei in a 50 μ l volume. After 60 minutes the salt concentrations etc were raised to those of an incubation assay. 10 μ l samples were taken at 0 and 30 minutes of the following incubation. As controls, nuclei were preincubated in the same way with buffer, egg extract and the normal oocyte extract (=whole ovary extract).

bacterial contamination increases with time, so a preincubation time of 60 minutes followed by an incubation of 30 minutes was standardly used. Nuclei remain transcriptionally active during an incubation of this time [FIG 3.9].

The method of spinning and resuspending the nuclei between the preincubation and incubation steps does have the disadvantage of being cumbersome when more than 12 assays are being performed. In these cases, therefore, the preincubated nuclei were supplemented with salts, triphosphates etc to bring the concentrations up to those of the standard assay buffer. This variation yields the same increase in incorporation as seen with the spun and resuspended nuclei [TABLE 3.3].

Table 3.4 shows that if the oocyte extract is made from separated and defolliculated vitellogenic oocytes and not from the usual whole ovary preparation, only a slight increase is seen in incorporation over that produced by the standard preparation. It would seem likely therefore, that whatever is causing this increase in incorporation is present in the oocytes themselves and not in the surrounding follicle cells, other non-oocyte ovarian tissue or in small, rather than vitellogenic oocytes. The slightly higher stimulation is presumably because more of this oocyte component is present per mg protein in the separated oocyte preparation.

Actinomycin D is a potent inhibitor of RNA polymerases. At a concentration of 100 $\mu\text{g/ml}$ it will inhibit all three of the RNA polymerases (Widnell and Tata, 1966). It acts not by binding to the RNA polymerase molecule itself, as α -amanitin does (Jacob

TABLE 3.5

Inhibition of Transcription by Actinomycin D.

	pmoles UMP incorporated/ 10^6 nuclei/30 mins.	
	- Actinomycin D	+ Actinomycin D
Nuclei + buffer	5.3	0.02
egg extract	10.7	0.01
oocyte extract	31.7	0.00

Nuclei were preincubated with the relevant protein extract and with or without 100 μ g/ml Actinomycin D. After 60 minutes the nuclei were spun out and taken up in a standard incubation mix, with Actinomycin D where appropriate. 10 μ l samples were taken for processing at 0 and 30 minutes of the incubation.

et al, 1970), but by binding to the DNA (Widnell and Tata, 1966). It is therefore possible to distinguish between transcription using a DNA template and that using endogenous RNA as template. The latter event has been reported for several in vitro transcription systems (Zasloff and Felsenfeld, 1977; Maryanka et al, 1979). These, however, were using E.coli RNA polymerase for transcription, rather than an homologous or endogenous enzyme and template. As Table 3.5 shows, Actinomycin D at 100 $\mu\text{g/ml}$ results in total inhibition of transcription by erythrocyte nuclei, either assayed on their own, or with egg or oocyte extracts.

Therefore, the incorporation of ^3H -UTP seen is not due to transcription from a RNA template but is due to DNA-dependent RNA polymerase activity. Neither is the increase in incorporation seen in oocyte-treated nuclei merely the sticking of ^3H -UTP to the filter by the extra protein, since this incorporation is sensitive to Actinomycin D.

RNA Polymerase Levels in Erythrocyte Nuclei Treated with Egg and Oocyte Extracts.

Although these basic mixing experiments demonstrate that a crude oocyte extract brings about an increase in incorporation of ^3H -UTP into acid-insoluble material, they do not give any insight into the relative activities of the three RNA polymerases. For this the specific inhibitor α -amanitin has to be used to differentiate between two possibilities. Either the endogenous RNA polymerase II of the erythrocyte nuclei is elongating 6 fold faster as a result of some component in the oocyte extract, or the other RNA polymerases in the extracts have initiated and

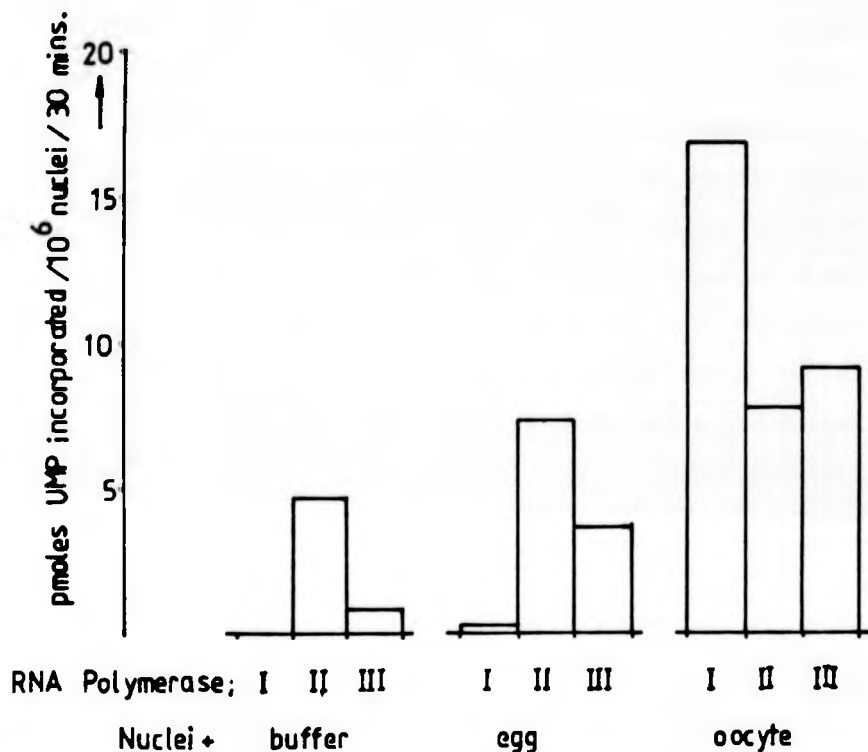


FIGURE 3.12

Relative Levels of the Three RNA Polymerases in Extract-Treated Erythrocyte Nuclei.

Erythrocyte nuclei were preincubated (5×10^5 nuclei in 50 μ l assay) with buffer, egg extract or oocyte extract and with α -amanitin at 0, 1 or 100 μ g/ml. These assays were supplemented after 60 minutes to incubation concentrations and incubated for a further 30 minutes. Duplicate 10 μ l samples were taken at 0 and 30 minutes of this incubation.

started to transcribe from the erythrocyte chromatin, those in the oocyte extract doing so more than those in the egg extract.

The levels of all three RNA polymerases in nuclei treated with both oocyte and egg extracts were determined using α -amanitin and compared to the levels found in untreated nuclei [FIG 3.12]. As can be seen, preincubation with an egg extract results in an increase in both RNA polymerase II and III activity (to 7.15 and 3.59 pmoles UMP incorporated/ 10^6 nuclei/30 minutes respectively) There is also some α -amanitin insensitive RNA polymerase I activity detectable (0.26 pmoles UMP incorporated/ 10^6 nuclei/30 minutes), but this is at a level barely above background. If, on the other hand, the nuclei are preincubated with an oocyte extract, then all three RNA polymerases show increased activity. In the case of RNA polymerase II it is to the same level as is shown by egg-treated nuclei, whilst RNA polymerase III shows an increase in activity (to 9.01 pmoles UMP incorporated/ 10^6 nuclei /30 minutes) above that given by egg-treated nuclei. However, these increases in the activities of RNA polymerases II and III do not account for more than 49.9 % of the transcription in oocyte-treated nuclei, the remaining 50.1% is due to RNA polymerase I activity. Since there is no detectable RNA polymerase I activity in the erythrocyte nuclei when assayed alone, it is reasonable to assume that the RNA polymerase I molecules came from the oocyte extract and that the transcription seen is as a result of the initiation of new RNA chains on the erythrocyte chromatin. That this is so can be shown in two ways. Firstly by using an inhibitor of RNA polymerase initiation it should be possible to inhibit all RNA polymerase I activity in the oocyte-treated nuclei; secondly by demonstrating that there are indeed no RNA polymerase I molecules present in the

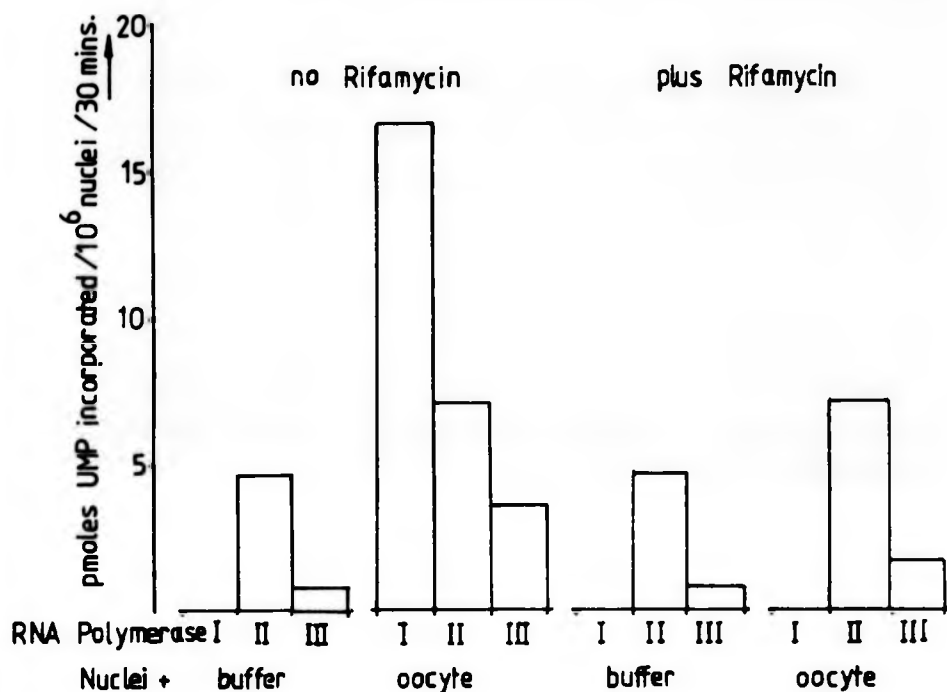


FIGURE 3.13

Inhibition of Transcription by Erythrocyte Nuclei by Rifamycin AF/013.

Erythrocyte nuclei (2×10^5 nuclei in 50 μ l) were preincubated with 20 μ l of column buffer or oocyte extract, with and without 50 μ g/ml Rifamycin AF/013, and 0, 1 or 100 μ g/ml α -amanitin. The assays were supplemented to produce the standard incubation conditions after 60 minutes preincubation and duplicate 10 μ l samples taken at 0 and 30 minutes of the following incubation.

erythrocyte nuclei. These latter would have to be bound very tightly to the chromatin, so that under normal assay conditions no elongation occurs. Some component of the oocyte extract would therefore have to weaken this binding to allow transcription to proceed.

Whilst the Rifamycin is a potent inhibitor of bacterial RNA polymerase (Hartmann et al., 1967), it has no effect on eukaryotic RNA polymerases (Jacob et al., 1968). However, several of its synthetic derivatives do inhibit these enzymes. In particular, the Rifamycin SV derivative AF/013 has been shown (Meilhac et al., 1972) to inhibit RNA polymerases I and II at initiation, whilst not inhibiting already initiated and elongating RNA polymerase molecules. If this inhibitor is added to erythrocyte nuclei with or without oocyte extract [FIG 3.13] it can be seen that RNA polymerase II activity is not altered either in untreated or oocyte-treated nuclei. RNA polymerase I activity, on the other hand, is completely inhibited in oocyte-treated nuclei. This again shows that the RNA polymerase II activity seen in erythrocyte nuclei is due to elongation of a preinitiated nascent RNA molecule. The stimulation in RNA polymerase II seen on the addition of an oocyte or egg extract is due to an increase in the rate of elongation of the final RNA transcript and not reinitiation or new initiation by the RNA polymerase II molecules from the protein extracts. About 48% of the RNA polymerase III transcription seen in oocyte-treated nuclei is due to previously initiated molecules, which show an increased rate of elongation, whilst the remainder is due to newly initiated molecules. The RNA polymerase I activity seen in oocyte-treated nuclei, however, is completely sensitive to Rifamycin AF/013 inhibition. This would indicate that it is due

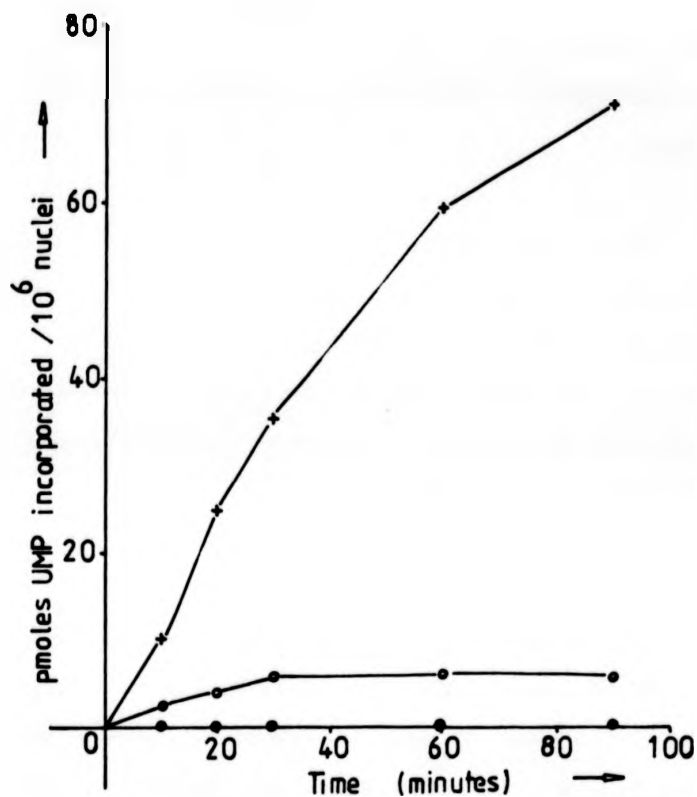


FIGURE 3.14

Effect of Sarkosyl on Transcription by Erythrocyte Nuclei.

Three 150 μ l transcription assays were set up (1.5×10^6 nuclei/assay) with nuclei alone (o---o), nuclei with 1% Sarkosyl (+---+) or nuclei with 1% Sarkosyl and 100 μ g/ml α -amanitin (●---●). Duplicate 10 μ l samples were taken at intervals throughout the timecourse.

TABLE 3.6

Necessity of Active RNA Polymerases During Preincubation.

Preincubation conditions		pmoles UMP incorporated	
Extract	α -amanitin	/10 ⁶ nuclei/30 mins.	
		- α -amanitin	+ α -amanitin
buffer	none	5.7	0.00
egg	none	11.3	0.23
oocyte	none	35.1	17.6
oocyte	1 μ g/ml	34.9	17.4
oocyte	100 μ g/ml	36.3	18.2
oocyte	none, no added XTP	27.2	13.7

Erythrocyte nuclei were preincubated with protein extracts, α -amanitin and triphosphates as above, for 60 minutes. After this time the nuclei were spun out and resuspended in a standard incubation mix with or without 100 μ g/ml α -amanitin to determine the RNA polymerase I activity. Samples were taken at 0 and 30 minutes of the incubation.

exclusively to initiation by RNA polymerase I molecules from the oocyte extract.

The anionic detergent, Sarkosyl, acts to remove nearly all the chromatin-associated proteins from DNA, except for initiated RNA polymerases (Green et al., 1975). Its effect is to block new initiation, but to facilitate the run off of pre-existing transcription complexes. When 1% Sarkosyl was added to erythrocyte nuclei there was an increase in transcriptional activity [FIG 3.14] but this was all due to run off by RNA polymerases II and III. It did not result in any measurable RNA polymerase I activity. Thus it would seem that the RNA polymerase activity in these erythrocyte nuclei is due to already initiated RNA polymerase II and III molecules whose elongation is inhibited by Sarkosyl-sensitive proteins, as described by Hentschell and Tata (1978). Further, there seem to be no RNA polymerase I molecules. The removal of almost all of the proteins associated with the chromatin does not result in the reactivation of any silent population of RNA polymerase I molecules.

By preincubating the nuclei with an oocyte extract plus a high (100 $\mu\text{g/ml}$) or a low (1 $\mu\text{g/ml}$) level of α -amanitin it is possible to determine whether the RNA polymerase I activity is dependent on either RNA polymerase II and/or RNA polymerase III molecules being active during the preincubation stage. As Table 3.6 shows, neither RNA polymerase II nor RNA polymerase III activity during the preincubation is necessary for subsequent RNA polymerase I activity during the assay. If on the other hand, all of the triphosphates are left out of the preincubation mix, but added later, this does have a small effect on the transcriptional activity of oocyte-treated erythrocyte nuclei. There is still a

stimulation observed over the ^3H -UTP incorporated by untreated or egg-treated nuclei [TABLE 3.6], but this is slightly less than that seen in oocyte-treated nuclei preincubated with the triphosphates.

Thus the RNA polymerase I activity observed in oocyte-treated nuclei is not dependent on prior RNA polymerase II or III activity, neither is it totally dependent on added triphosphates during the low salt preincubation stage. This effect might be small because there is a triphosphate pool in the oocyte extract itself. The likely explanation of the higher synthesis with added triphosphates is that they permit the loading of many RNA polymerase I molecules on to the activated ribosomal genes.

RNA Polymerase Levels in Oocyte and Egg Extracts.

It could be argued that the stimulation observed when erythrocyte nuclei are incubated with an oocyte extract is just a reflection of the RNA polymerases present in the extract. An egg extract might give a lower stimulation because it contains no RNA polymerase I. Roeder (1974b) has shown that the levels of the three RNA polymerases remain constant not only during the change from oocyte to egg, but also during the early development of the embryo. It is only at embryonic stage 30-33 that the relative amounts alter. During this time, the actual transcription by the RNA polymerases on embryonic nuclei varies significantly, from a high level of transcriptional activity during oogenesis, to no transcription at all in the unfertilized egg and early cleavage embryo. Non-rRNA synthesis has been detected as early as the blastula stage (Newport and Kirschner, 1982a), whilst the first

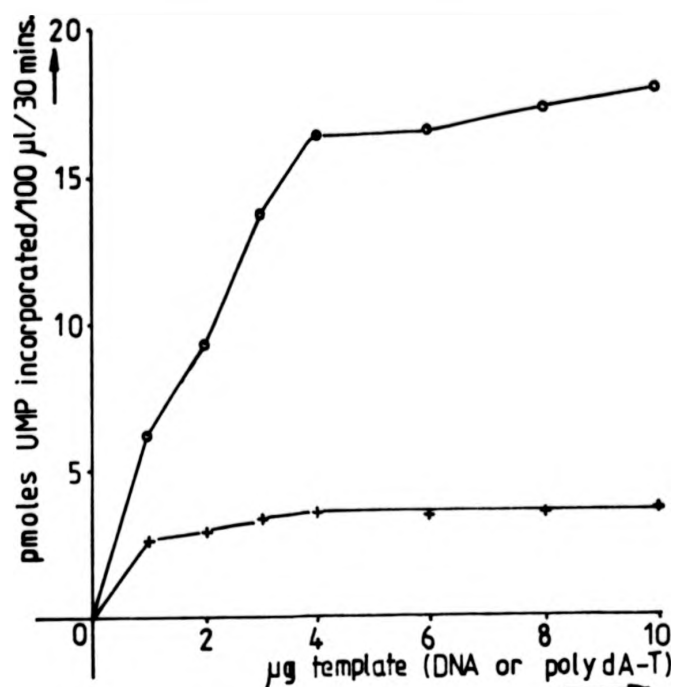


FIGURE 3.15

RNA Polymerase Activity in an Oocyte Extract.

12.5 µl of oocyte extract was incubated in a 50 µl assay under the same conditions as a nuclear transcription assay, but instead of nuclei, the template was increasing amounts of poly[d(A-T)] (o---o) or purified *X.laevis* DNA (+---+). Duplicate 10 µl samples were taken at 0 and 30 minutes of the assay.

TABLE 3.7

RNA Polymerase Levels in Oocyte and Egg Extracts.

Extract	Template	pmoles UMP incorporated
		/100 μ l assay/30 mins.
buffer	none	0.00
egg	none	0.00
oocyte	none	0.00
egg	4 μ g poly[d(A-T)]	120.0
oocyte	4 μ g poly[d(A-T)]	167.7

The extracts were incubated in a standard incubation mix, but without nuclei, 4 μ g poly[d(A-T)]/100 μ l assay was added as required. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the incubation period.

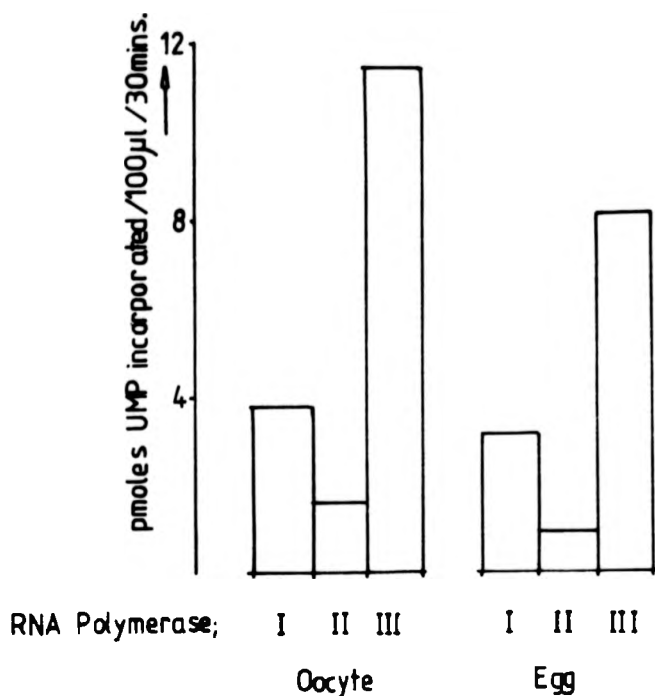


FIGURE 3.16

RNA Polymerase Levels in Oocyte and Egg Extracts.

50 µl transcription assays were set up containing 2 µg poly[d(A-T)], 20 µl oocyte or egg extract and 0, 1 or 100 µg/ml α -amanitin. These were incubated for 30 minutes, samples (2x10 µl) being taken at the beginning and end of this period.

rRNA synthesis has been noted in mid to late blastula stage embryos (Shiokawa et al, 1981a, b). RNA polymerase assays were performed to check that the relative levels of the three RNA polymerases were identical in both oocyte and egg extracts. Exactly the same conditions were used as for the nuclear transcription assays, thus ensuring that the measurements taken accurately reflected the levels of the RNA polymerases that were active under the normal assay conditions. However, instead of using X.laevis DNA as the template for the RNA polymerases, the synthetic template poly[d(A-T)] was used. This gave a better incorporation of ³H-UTP into acid-insoluble material [FIG 3.15]. A concentration of 40 µg/ml poly[d(A-T)] was used standardly in all the following assays. The values obtained from these assays for the levels of the three RNA polymerases might be different from those determined by Roeder (1974a, b) since the extraction procedure and subsequent assay conditions are not the same.

As Table 3.7 shows, neither egg nor oocyte extract incubated in an assay system without a template results in any transcription. When poly[d(A-T)] is added, both extracts show high total RNA polymerase activity, but the egg is only 70% as active as that of the oocyte. However, the relative amounts of the three RNA polymerases are similar in both extracts [FIG 3.16]. Thus in both egg and oocyte extracts, 67% of the total RNA polymerase activity observed with poly[d(A-T)] as template is as a result of RNA polymerase III, a further 25% is due to RNA polymerase I and the remaining 8% as a result of RNA polymerase II activity. The RNA polymerase activity seen in oocyte-treated nuclei cannot therefore be a simple reflection of the RNA polymerase levels in the extract. RNA polymerase I represents only 25% of the active RNA polymerases in the oocyte extract

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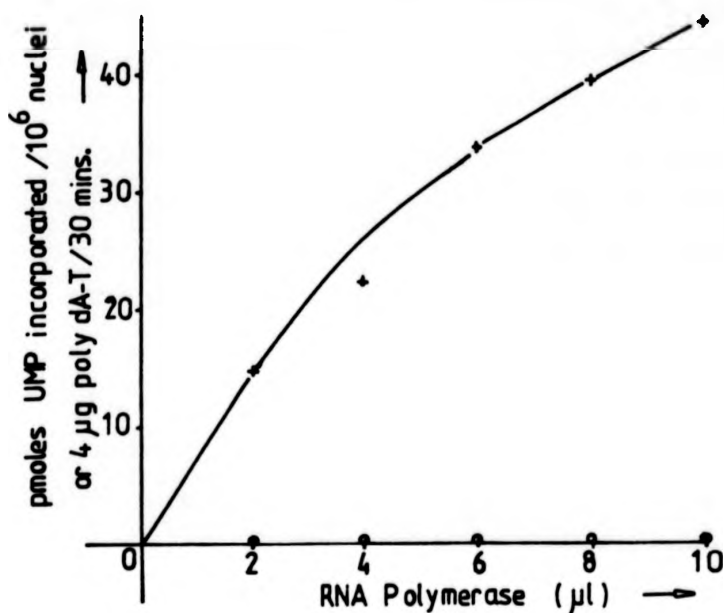


FIGURE 3.17

Transcription on Erythrocyte Chromatin or poly[d(A-T)] Produced by Added, Semi-purified RNA Polymerases.

Pairs of 50 μ l transcription assays were set up with either 5×10^5 nuclei (o---o) or 2 μ g poly[d(A-T)] (+---+) and increasing amounts of a semipurified RNA polymerase preparation. 100 μ g/ml α -amanitin was included in all the assays, such that only RNA polymerase I activity is seen. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the transcription assay.

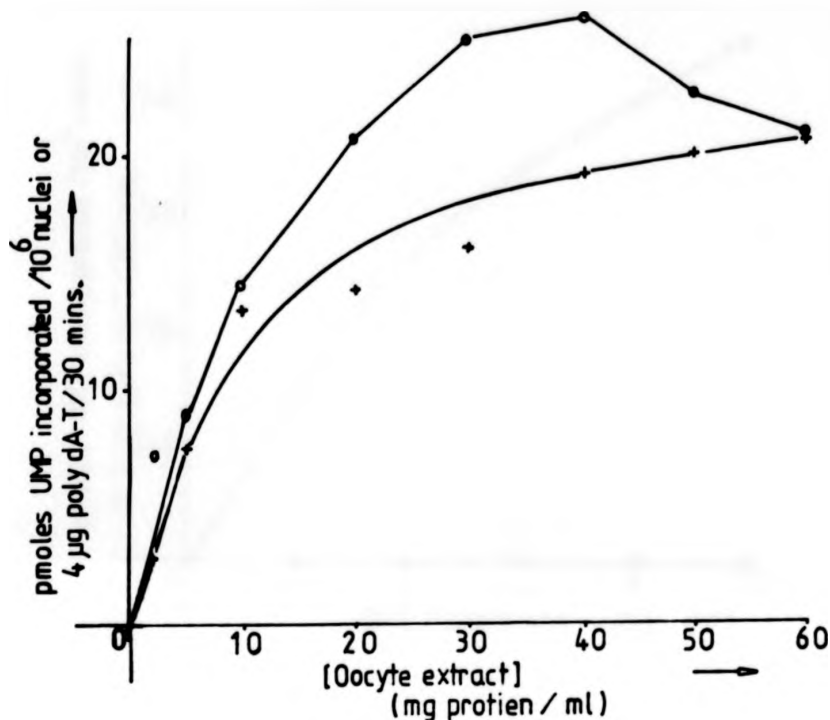


FIGURE 3.18

Effect of Increasing the Concentration of Oocyte Extract in the Transcription Assay.

Increasing concentrations of oocyte extract (from 5 to 60 mg protein/ml) were incubated for 30 minutes in a 50 μ l assay with either 5×10^5 nuclei (o---o) or 2 μ g poly[d(A-T)] (+---+). 2×10^4 μ l samples were taken at 0 and 30 minutes of the incubation.

under these assay conditions, but transcribes 50% of the RNA made by erythrocyte nuclei added to this extract. Since all three RNA polymerases are present in the egg extract, it is not lack of RNA polymerase I which causes the lack of stimulation seen in egg-treated nuclei, but rather the lack of some other component of the oocyte extract or possibly the presence of an inhibitor, which affects its activity on nuclei but not on poly[d(A-T)].

Effect of RNA Polymerase I on Erythrocyte Nuclear Transcription.

Another indication that the increased activity seen in oocyte-treated nuclei is not a reflection of the mere presence of RNA polymerase I, is given by the addition of a crude RNA polymerase preparation to the nuclei. As can be seen from Figure 3.17, increasing the amount of RNA polymerase I has no effect on nuclear transcription at all, whereas with poly[d(A-T)] as template there is a corresponding increase in incorporation of ³H-UTP into acid-insoluble material. If, however, the amount of oocyte extract is increased in a nuclear assay, an increase in incorporation can be observed [FIG 3.18]. Starting with a concentrated oocyte extract and diluting it with column buffer, it can be seen that decreasing the amount of oocyte extract results in a decrease in transcription by the nuclei. Above 40 mg/ml of protein, however, the extract does not elicit any further response, and above 50 mg/ml protein, addition of the extract shows a decreased response. If the nuclei are replaced by poly[d(A-T)] in this assay, [FIG 3.18], the system becomes saturated above 40 mg/ml of protein, but shows no decreased response above this concentration.

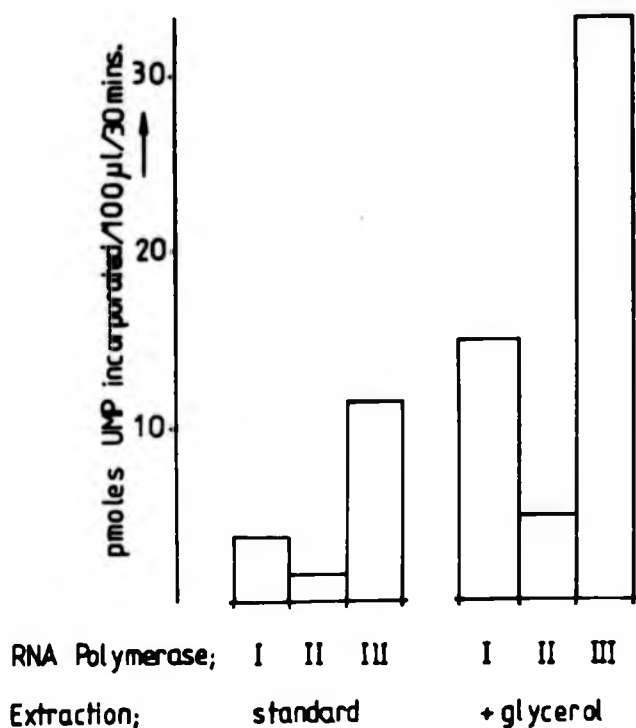


FIGURE 3.19

RNA Polymerase Levels in Oocyte Extracts.

12.5 µl of a standard oocyte extract, or one extracted using a glycerol-rich buffer were incubated with 2 µg poly[d(A-T)] in a 50 µl assay with 0, 1 or 100 µg/ml α -amanitin. These were incubated for 30 minutes with 2x10 µl samples being taken at 0 and 30 minutes.

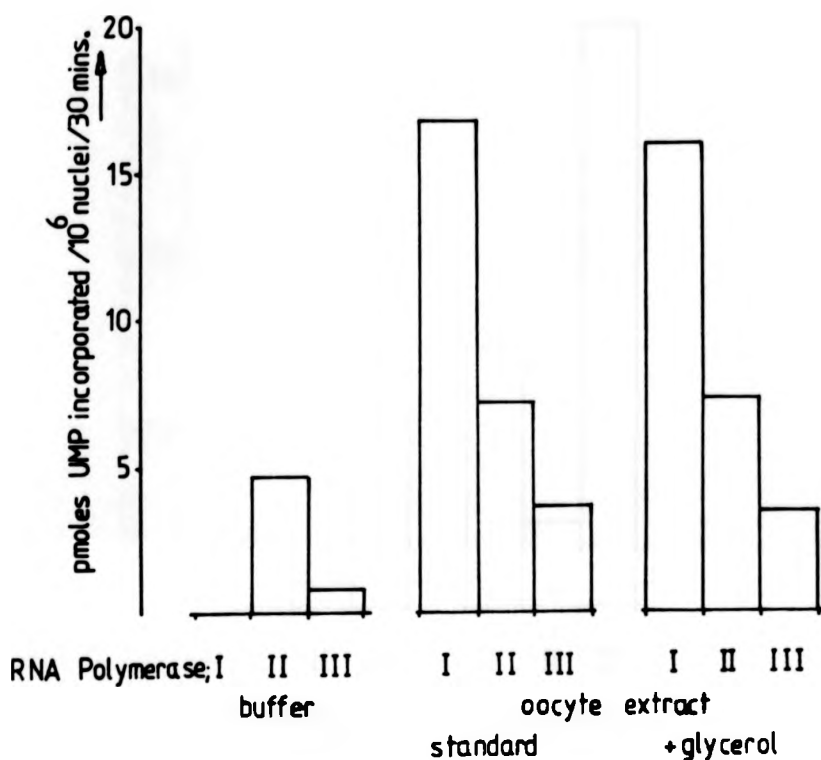


FIGURE 3.20

RNA Polymerase Levels in Erythrocyte Nuclei Treated with Oocyte Extracts.

20 μ l of column buffer, a standard oocyte extract, or one extracted using a glycerol-rich buffer were preincubated for 60 minutes with 5×10^5 nuclei in a 50 μ l assay with 0, 1 or 100 μ g/ml α -amanitin. After salt supplementation, the nuclei were incubated for a further 30 minutes, duplicate 10 μ l samples were taken at 0 and 30 minutes of the incubation stage.

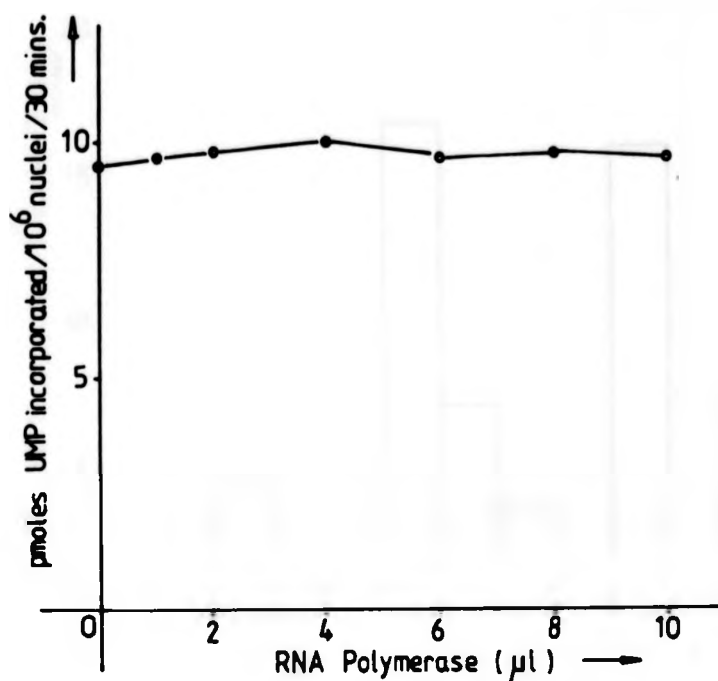


FIGURE 3.21

Addition of Extra RNA Polymerase I to Erythrocyte Nuclei
Supplemented with Oocyte Extract.

To 50 μ l assays containing 5×10^5 nuclei, 10 μ l oocyte extract and 100 μ g/ml α -amanitin were added increasing amounts of the crude RNA polymerase preparation. These were incubated for 60 minutes, supplemented to give the usual final incubation conditions, and then incubated for 30 minutes. Samples (2 \times 10 μ l) were taken at 0 and 30 minutes of this incubation.

To see whether the amount of RNA polymerase I in the oocyte extract is in any way rate-limiting, two oocyte extracts were made in parallel. Both started with the same number of oocytes and finished with the same volume; one was made using the standard extraction process, the other using a glycerol-rich extraction buffer thus maximising the RNA polymerase content. This second extraction procedure results in a higher level of RNA polymerase activity [FIG 3.19], but it does not result in an increase in the amount of RNA polymerase I activity seen when this extract is added to erythrocyte nuclei [FIG 3.20]. Extra RNA polymerases can also be added in the form of a semipurified RNA polymerase preparation. By adding increasing amounts of this in the presence of the inhibitor α -amanitin, such that only RNA polymerase I activity is possible, it can be shown [FIG 3.21] that excess, semipurified RNA polymerase I has no effect.

Although the response of nuclei is dependent on the amount of extract added, this effect is not changed by adding extra RNA polymerases. Thus some other part of the oocyte extract must always be rate limiting. It would seem likely, therefore, that there is a component of the oocyte extract that induces RNA polymerase I activity in erythrocyte nuclei. It is distinct from the RNA polymerase I molecule itself, since incubation of the nuclei with extra RNA polymerases does not elevate RNA synthesis by RNA polymerase I. This component has not been detected in egg extracts.

If this explanation, in terms of an activator present in oocytes, but not eggs, is correct, it should be possible to dilute the oocyte extract with an egg extract and achieve the same amount of transcription as if a neutral agent like a column

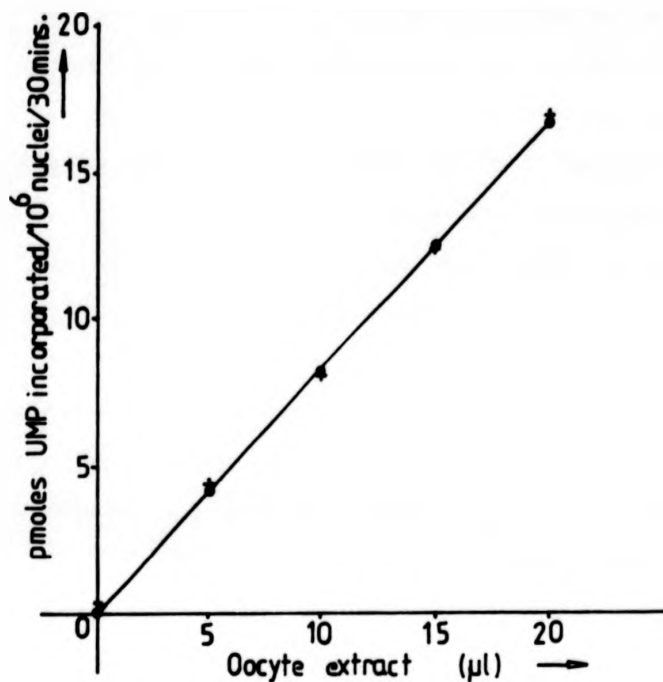


FIGURE 3.22

Dilution of Oocyte Extract by Column Buffer and Egg Extract.

5×10^5 nuclei in a 50 μ l assay were preincubated with 20 μ l of oocyte extract diluted with either column buffer (o---o) or an egg extract (+---+), and 100 μ g/ml α -amanitin. After this the assays were supplemented with salts etc and incubated for 30 minutes. Samples (2x10 μ l) were taken at the beginning and end of this incubation period.

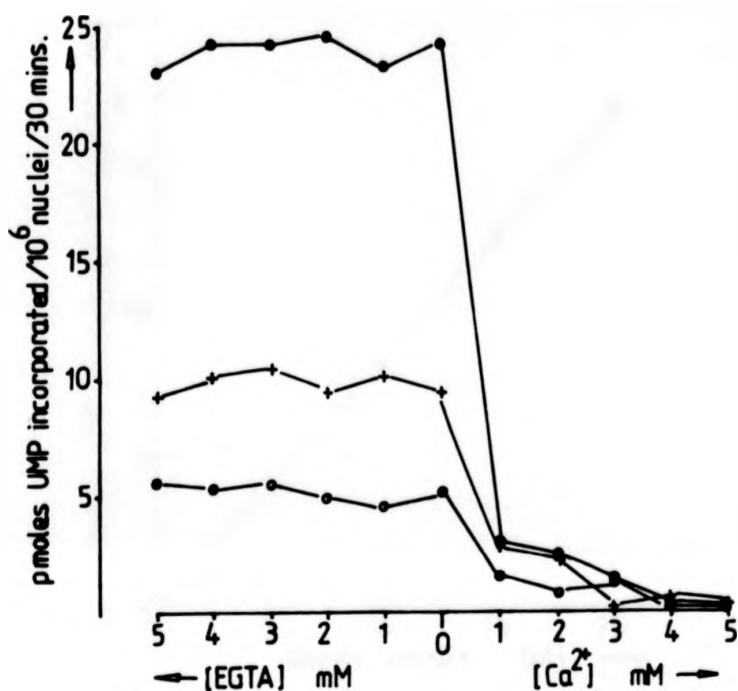


FIGURE 3.23

Effect of Calcium and EGTA on Transcription by Erythrocyte Nuclei.

5×10^5 nuclei in a 50 μ l assay were incubated with 12.5 μ l of buffer (o---o), egg extract (+---+) or oocyte extract (●---●) and increasing amounts of CaCl or EGTA (0 to 5 mM). These were incubated for 30 minutes, samples (2 \times 10 μ l) being taken at 0 and 30 minutes.

buffer had been used. If, on the other hand, there is an excess of an inhibitor present in the egg extract, as well as the oocyte activator-molecule, the egg extract would produce a greater decrease than that shown on dilution by buffer. As previously mentioned, a simple assay with erythrocyte nuclei will only detect an activator. Figure 3.22 shows that the dilutions of oocyte extract with either buffer or egg extract produce identical results on erythrocyte nuclei. Therefore there can be no excess of inhibitor in the egg extract.

Calcium and Transcription by Erythrocyte Nuclei.

Calcium is known to be involved in the regulation of many cellular processes, including key events of early development (Steinhart et al., 1977; Cuthbertson et al., 1981). So the difference between the oocyte and egg extract could be due to the presence and absence of calcium. Calcium has a detrimental effect on transcription by erythrocyte nuclei [FIG 3.23]. EGTA, which specifically binds to calcium, has no effect on transcription. Neither of the two extracts overcome this inhibition by calcium, and EGTA does not increase or decrease the amount of transcription in the treated nuclei. Calcium, therefore, does not appear to be the cause of the difference between the effect of oocyte and egg extracts on erythrocyte nuclear transcription.

Change in Size of Erythrocyte Nuclei by Oocyte Extract.

The mature erythrocyte nucleus is small compared with nuclei from earlier stages of erythropoiesis or other more transcriptionally active nuclei. Its 6 pg of DNA become tightly packaged and condensed during its development into the picnotic

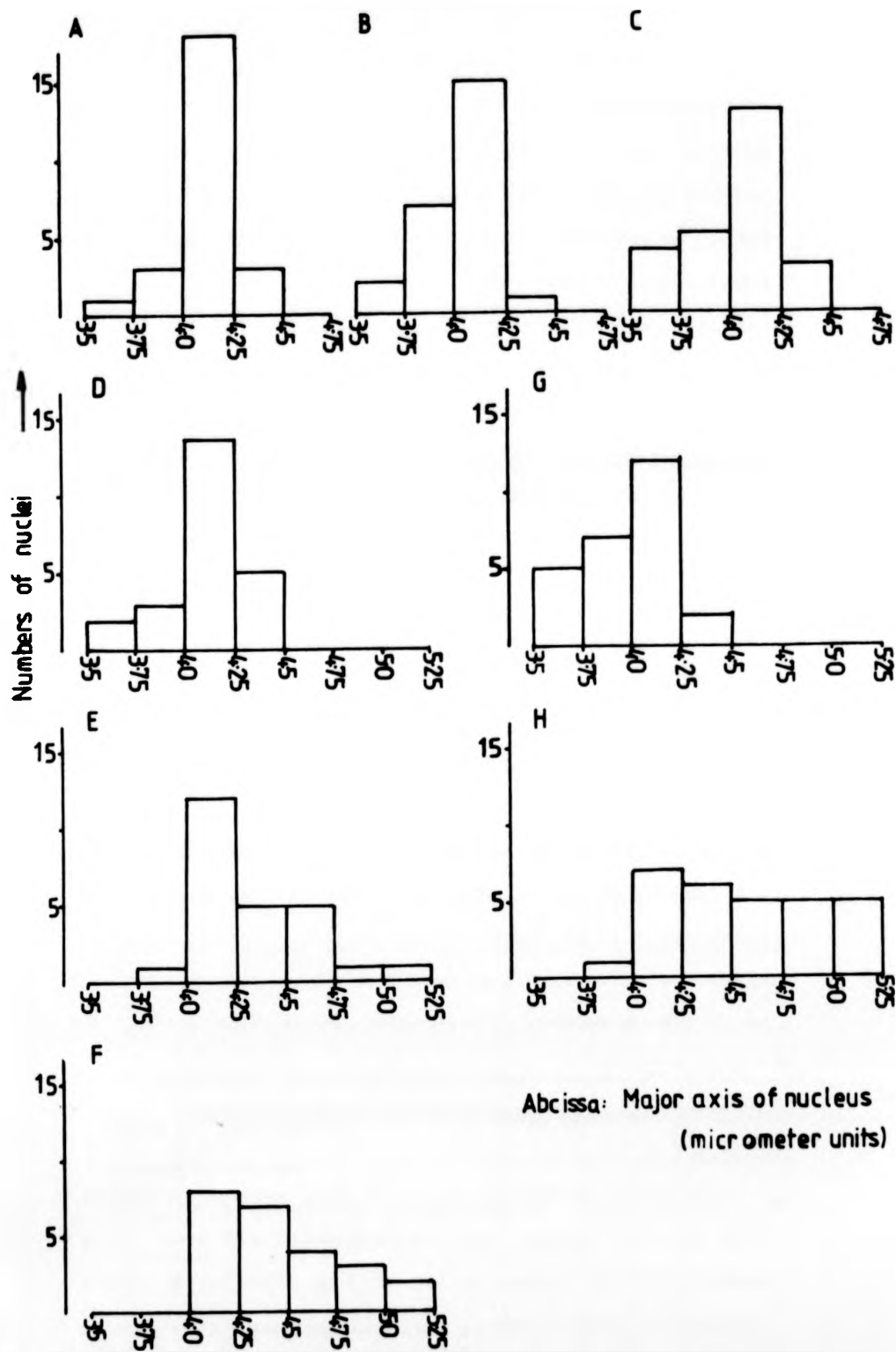


FIGURE 3.24

Change in Dimensions of Erythrocyte Nuclei on Incubation with Oocyte Extract.

Erythrocyte nuclei (5×10^5 nuclei/50 μ l) were incubated with 12.5 μ l of buffer, egg extract or oocyte extract. After 90 minutes for the buffer and egg extract incubates, and 15, 45 and 90 minutes for the oocyte extract, a 5 μ l sample was removed, diluted into fresh RB and the dimensions of 25 nuclei determined. Nuclei were also preincubated with buffer or oocyte extract (5×10^5 nuclei, 20 μ l extract/50 μ l), spun out after 60 minutes and resuspended in RB before being measured. The dimensions quoted are for the long axis and are micrometer units where 1 micrometer unit = 2.5 μ m.

	Extract	Time (minutes)
A	buffer	0
B	buffer	90
C	egg	90
D	oocyte	15
E	oocyte	45
F	oocyte	90
G	buffer	60 (preincubation)
H	oocyte	60 (preincubation)

nucleus found in the mature erythrocyte. It might be supposed that for transcription to occur, the DNA must be in a more dispersed state to allow the RNA polymerases free access to the relevant gene sequences. This opening up of the chromatin can be seen when somatic cell nuclei are injected into oocytes (Gurdon, 1976; Gurdon *et al*, 1976; Korn and Gurdon, 1981), these undergo a several hundred fold increase in size that is correlated with an increase in transcription. To see whether the erythrocyte nuclei incubated in vitro in these assays with an oocyte extract also show an increase in nuclear size, their size was determined under phase microscopy. Erythrocyte nuclei incubated without any extract did not alter their size (of $10 \times 7 \mu\text{m}$) over a 90 minute incubation period [FIG 3.24]. Nuclei incubated with an oocyte extract though, even after a short space of time (15 minutes), show an increase in size. A maximum size of $11 \times 8 \mu\text{m}$ is reached after 45 minutes and is not increased thereafter. If the nuclei are preincubated for 60 minutes, spun and resuspended in extract-free incubation buffer, the mean size remains the same during the incubation. Nuclei treated with an egg extract do not show an increase in size [FIG 3.24] when measured in the same way as oocyte-treated nuclei. Thus the oocyte extract brings about an increase of about 39% in the mean volume of the nuclei. Furthermore, there appears to be a shift in the size of all of the nuclei, and not a small population. This increase in size does not need the continued presence of the bulk oocyte extract for its maintenance, although presumably some molecules pass into the nuclei from the oocyte extract to bring about this size increase.

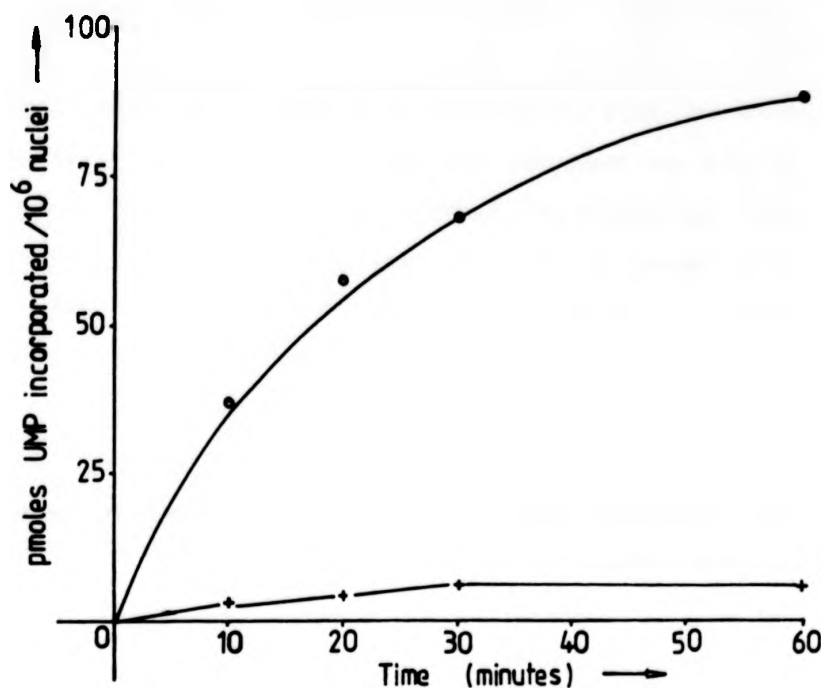


FIGURE 3.25

Transcription by *Xenopus laevis* Liver Nuclei.

Liver nuclei were assayed for transcriptional activity in a standard incubation assay (1.5×10^6 nuclei/150 μ l). Duplicate 10 μ l samples were taken at intervals throughout the timecourse (o---o). An identical timecourse with erythrocyte nuclei was also carried out in parallel (+---+).

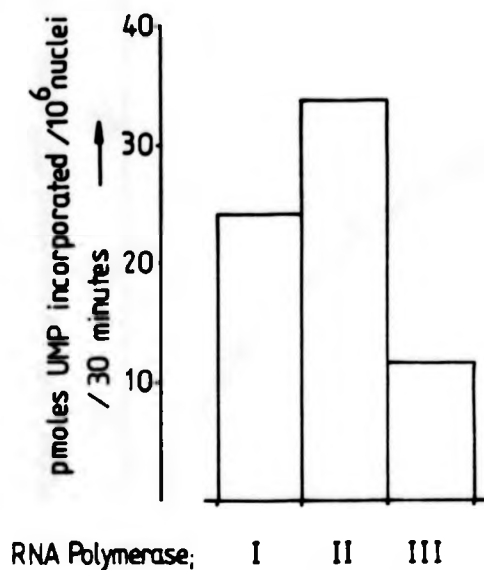


FIGURE 3.26

Relative Levels of the Three RNA Polymerases in Liver Nuclei.

To 50 μ l assays containing 5×10^5 liver nuclei, were added, either 0, 1 or 100 μ g/ml α -amanitin. These were incubated for 30 minutes and duplicate 10 μ l samples taken at the beginning and end of the incubation.

TABLE 3.6

Transcription by Liver Nuclei Incubated with Protein Extracts.

	pmoles UMP incorporated /10 ⁶ nuclei/30 mins.
Nuclei + buffer	66.6
egg extract	66.0
oocyte extract	69.4

Liver nuclei were incubated in the above extracts in a standard incubation assay. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the incubation period.

Transcription in Liver Nuclei.

Whereas transcription in the nuclei of intact erythrocytes is confined to the slow elongation of the final RNA transcripts, the liver transcribes a great variety of RNA species at a high rate using all three RNA polymerases. As a consequence, [FIGS 3.25, 3.26] untreated, isolated liver nuclei are about 12 to 14 times as active in RNA synthesis as erythrocyte nuclei, 30.1% of this being due to RNA polymerase I, 53.2% RNA polymerase II and 16.7% RNA polymerase III. The liver nuclei were used to study the effect of oocyte extract on rRNA genes that were already active. This contrasts with the situation in erythrocyte nuclei, where the extract must activate the genes. Oocyte and egg extracts were added to liver nuclei, after a 60 minute preincubation period the nuclei were spun out and resuspended in the standard incubation buffer. Incorporation of ^3H -UTP into acid-insoluble material was measured after 30 minutes. As Table 3.8 shows, neither extract brings about any change in the transcription by liver nuclei. The oocyte extract component that brings about transcription by RNA polymerase I on an inactive genome, has no effect on actively transcribed ribosomal genes and their accompanying RNA polymerase I molecules. This component therefore, would not seem to affect either the rate of elongation of the ribosomal 40S precursor molecule being transcribed or the rate of initiation of the RNA polymerase I molecules on the rDNA of liver nuclei. It could be that liver nuclei already have an excess of the relevant component, or this component acts on the inactive gene itself to make it accessible in some way to the RNA polymerase I molecules such that transcription can commence.

Summary

The erythrocyte nuclei of Xenopus laevis are transcriptionally inactive, in that when they are placed in a transcription system that has been adjusted to give the maximum incorporation of ^3H -UTP into acid insoluble material, they only incorporate 5.5 pmoles UMP/10 nuclei/30 minutes. Most of this transcription is due to elongation by RNA polymerase II, although some RNA polymerase III activity is detectable. When the nuclei are preincubated with a protein extract from oocytes or eggs under lower KCl and XTP concentrations, prior to the transcription assay itself, the total amount of UMP incorporated increases two-fold in the case of the egg extract and six-fold with the oocyte extract. With both extracts the rate of elongation of RNA polymerases II and III is increased, some new initiation by both of these polymerases is also seen. However, by far the largest contribution to the RNA polymerase activity in the oocyte-treated nuclei, is newly initiated RNA polymerase I. This transcription is from a DNA template, not from the RNA that is present in the extract, nor is it mobilization of a sequestered population of RNA polymerase I molecules within the nuclei. Since both oocyte and egg extracts contain the same relative amounts of each of the three RNA polymerases, the lack of RNA polymerase I activity seen in egg-treated nuclei is not a reflection of a lack of this polymerase in the egg extract. Transcription on an erythrocyte nuclear template is not effected by semipurified RNA polymerase I. An oocyte extract has no effect on transcription by liver nuclei that do transcribe their rRNA genes.

It would therefore appear that there is an activator molecule or molecules present in Xenopus laevis oocytes, that brings about transcription by RNA polymerase I on inactive rDNA. Either this molecule(s) is not present in unfertilized eggs, or it is not extracted under these conditions.

CHAPTER IV: LOCATION AND OCCURRENCE OF THE RIBOSOMAL ACTIVATOR DURING OOGENESIS AND EARLY EMBRYOGENESIS

RNA Transcription during Oogenesis.

The oocyte, as previously discussed (Chapter I) contains all the RNA and certain proteins necessary for the early development of the embryo. During oogenesis therefore, a great deal of energy has to be expended in order to synthesize these molecules. Enough ribosomes have been synthesized to last until the swimming tadpole stage - as seen by the continued survival of the anucleolar mutants to this stage without further rRNA synthesis (Brown and Gurdon, 1964; Knowland and Miller, 1970; Miller and Knowland, 1970). Ribosome production involves not only the transcription of the 18S and 28S rRNA species, but also that of the 5S rRNA. The necessary tRNAs must also be transcribed to deal with the large amount of protein synthesis that is carried out from fertilization onwards. Finally, the mRNAs for important structural proteins, such as the histones and actins necessary for cell division to take place, have to be transcribed.

In order to produce this quantity of RNA, the oocyte goes through a series of stages of differing RNA synthesis. The pre-vitellogenic oocyte synthesizes hnRNA, 4S (tRNA) and 5S RNA, but very little, if any, 40S rRNA (Ford, 1971; Mairy and Denis, 1972; Thomas, 1974). The 5S RNA is synthesized to such a degree that there is a 100 fold excess of 5S RNA over 18S and 28S RNA at this stage (Ford, 1971). The synthesis of these latter rRNA species from the extrachromosomal ribosomal DNA does not begin in earnest until early in vitellogenesis, and reaches a peak in mid-vitellogenesis (Ford, 1971). Although the rate of synthesis

thereafter slows down slightly, some rRNA synthesis occurs until oocyte maturation (Ford, 1971; LaMarca et al, 1973, 1975; Anderson and Smith, 1977) and the final meiotic division, when the amplified rDNA becomes dispersed and all RNA synthesis comes to a halt. 4S and 5S synthesis occurs throughout vitellogenesis, the finished molecules being stored in 7S and 42S ribonucleoprotein particles until they are needed (Ford, 1971; Picard et al, 1980). Presumably, in ribosome synthesis, the 5S DNA is the rate limiting component, and in order to have a sufficient supply of 5S rRNA for the vast numbers of ribosomes made during vitellogenesis the 5S genes have to be transcribed for a longer period (Mairy and Denis, 1972). There are 20,000 5Soc RNA genes/haploid genome (Brown et al, 1971) as compared to the 1.5 to 2.5 million copies of the rRNA genes (David et al, 1970). Messenger RNA species can be detected in all stages of oogenesis. Histone mRNA can be detected in pre-vitellogenic oocytes at levels comparable to those seen in vitellogenic oocytes, both by cell-free translation assays (Ruderman and Pardue, 1977) and by hybridization to cloned histone DNA sequences (van Dongen et al, 1981). Similarly the poly-A⁺ RNA species do not alter from those seen in the pre-vitellogenic oocyte (Rosbash and Ford, 1974; Darnborough and Ford, 1976; Golden et al, 1980). This is despite the fact that the lampbrush chromosomes do not appear until early vitellogenesis and are transcribed at a high rate (Anderson and Smith, 1978). Either this transcription does not go towards the maternal mRNA pool and is degraded, or serves some other undefined purpose, or the mRNA synthesized during early oogenesis has a limited half life and the lampbrush transcription serves to maintain the levels of the mRNA pool.

Throughout oogenesis, the relative activities of the three RNA polymerases remains constant (Roeder, 1974b) although in very early pre-vitellogenic oocytes, about the pachytene stage when ribosomal gene amplification is occurring (Perkowska et al., 1968; Gall, 1968; Kalt and Gall, 1974), the actual quantity of RNA polymerase is small. Between this stage and the mature oocyte there is a 500-fold increase in the amount of RNA polymerase (Roeder, 1974b). These unchanging relative levels of the three RNA polymerases are maintained even though each RNA polymerase has a different transcriptional activity at different stages of oogenesis. For instance, in pre-vitellogenic oocytes there are almost equal quantities of RNA polymerases I and III, but only the RNA polymerase III molecules are transcribing the oocyte chromatin, to produce 4S and 5S RNA. The RNA polymerase I molecules are idle, but it is not through lack of ribosomal genes. To the 48,000 5S genes available to the RNA polymerase III molecules (Brown et al., 1971), there are between 1.5 and 2.5 million copies of the ribosomal genes in the amplified rDNA (Perkowska et al., 1968). The lag between the appearance of the amplified rDNA (MacGregor, 1968; Gall, 1969; Kalt and Gall, 1974) and the onset of its transcription (Ford, 1971), even though RNA polymerase I is present in the oocyte (Roeder, 1974b), and the gradual increase in the transcription of these amplified genes to the maximal transcription of mid-vitellogenesis (MacGregor, 1968; Scheer, 1973), could be said to indicate the presence and gradual accumulation of some component within the developing oocyte. This component would be necessary for ribosomal gene transcription to occur and be quite separate and distinguishable from the RNA polymerase I molecules.

It was shown in Chapter III that a total ovary homogenate -

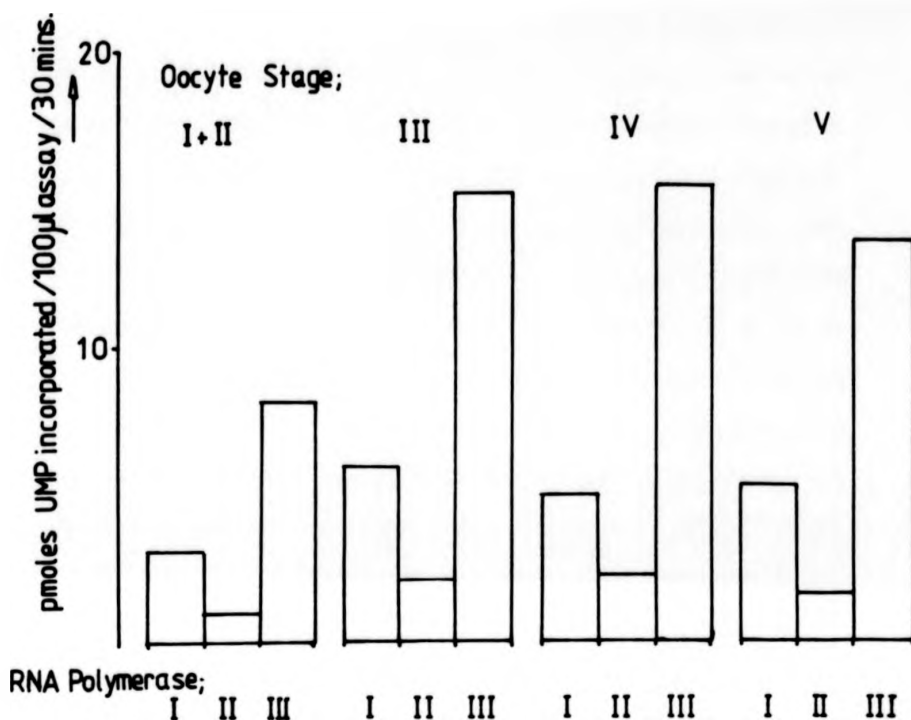


FIGURE 4.1

Relative Amounts of the Three RNA Polymerases During Oogenesis.

An ovary was stripped of its oocytes, which were sized before being washed in WH buffer. Protein extracts were prepared in the standard way from the various size classes and adjusted to 30 mg/ml. These extracts were assayed in the RNA polymerase assay using poly[d(A-T)] as template and the inhibitor α -amanitin to distinguish between the RNA polymerases (at 0, 1 and 100 μ g/ml). Duplicate 10 μ l samples were taken from the 50 μ l assay at 0 and 30 minutes.

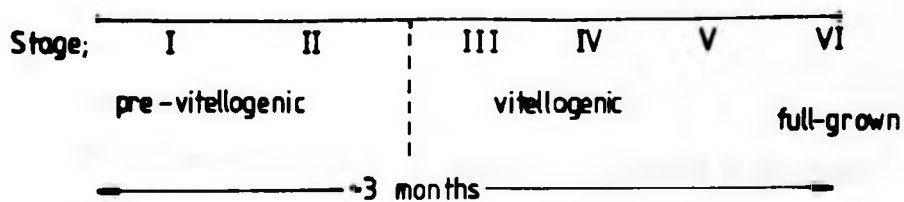
TABLE 4.1

RNA Polymerase Activity in Extracts of Oocytes of Different Stages.

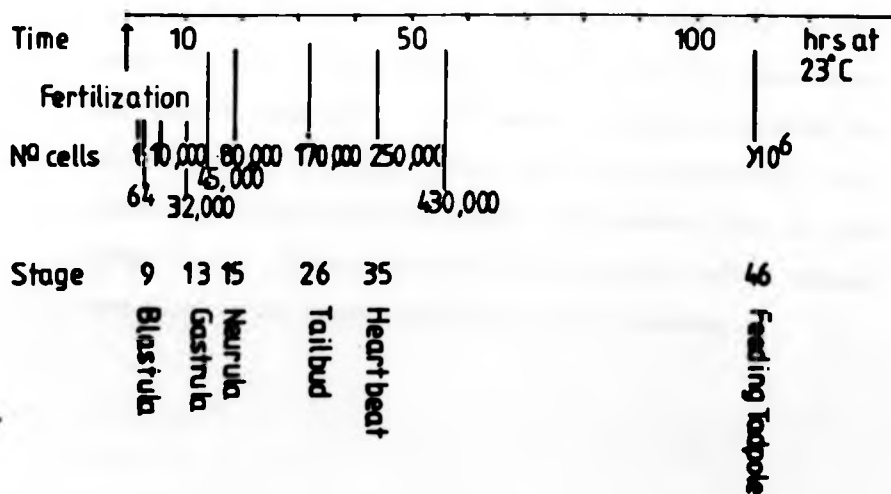
Oogenetic Stage:	pmoles UMP incorp. /1.2 mg protein/30 mins.	pmoles UMP incorp. /oocyte/30 mins.
I+II	12.3	0.0616
III	23.3	0.232
IV	22.7	0.76
V	20.4	1.36

The left hand column shows the results normalised to the amount of protein in the added extract, and the right hand column the results normalised to the number of oocytes from which the extract came. 50 μ l assays containing 2 μ g/ml poly[d(A-T)], 20 μ l oocyte extract from different stage oocyte extracts plus all the normal salts etc were incubated for 30 minutes. 2x10 μ l samples were taken at the beginning and end of the incubation.

Oogenesis:



Embryogenesis:



which will contain oocytes of all stages - contains a component that brings about an increase in transcription by RNA polymerase I of the inactive ribosomal RNA genes in erythrocyte nuclei. Since oocytes may be separated, according to size, it should be possible to see whether this component is present at all stages of oogenesis or only at a few.

RNA Polymerase Levels During Oogenesis.

When the relative levels of the three RNA polymerases were determined using the poly[d(A-T)] transcription assay, it was seen [FIG 4.1] that, although the percentage of the total activity for each RNA polymerase remained constant, the total amount of RNA polymerase activity increased during oogenesis, the pre-vitellogenic oocytes having 60% of the activity per mg protein seen in the full-grown oocytes. As these extracts were assayed on the basis of protein content and not oocyte numbers, it should be noted that more pre-vitellogenic oocytes went into the making of that extract than was the case with the mature oocytes. Therefore, if this is taken into account [TABLE 4.1], there is an increase (of 22-fold) in the total amount of RNA polymerase activity during oogenesis, ie the pre-vitellogenic oocytes (Stages I+II) contain only 4.5% of the RNA polymerase activity of the stage V vitellogenic oocytes. At all stages, in these extracts, RNA polymerase I constitutes about 25% of the total RNA polymerase activity.

Presence of the Ribosomal Gene Activator During Oogenesis.

If these oocyte extracts, derived from oocytes at different stages in their growth, are added to erythrocyte nuclei and

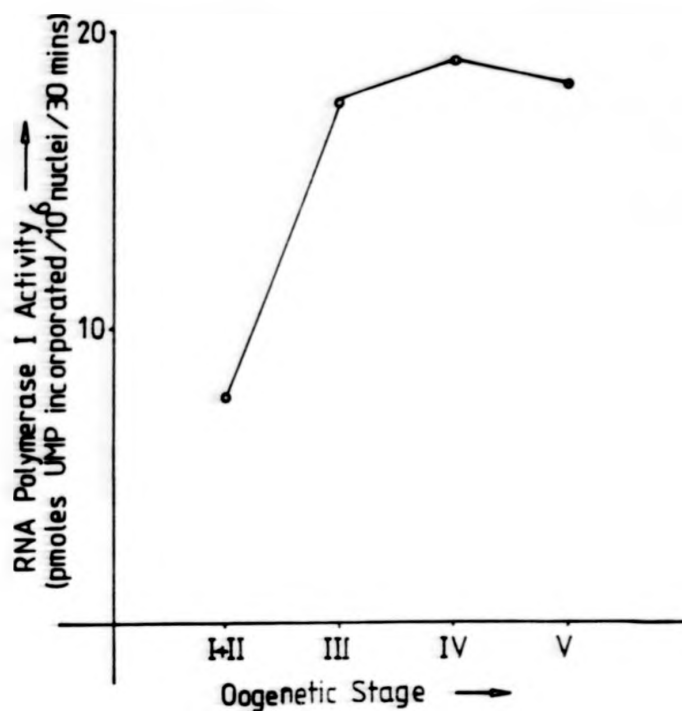


FIGURE 4.2

Effect of Oocyte Extracts from Different Stages of Oogenesis on RNA Polymerase I Transcription in Erythrocyte Nuclei.

The oocyte extracts described in Figure 4.1 were assayed with erythrocyte nuclei in a 50 μ l preincubation assay with 100 μ g/ml α -amanitin present such that only transcription by RNA polymerase I was possible. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the incubation.

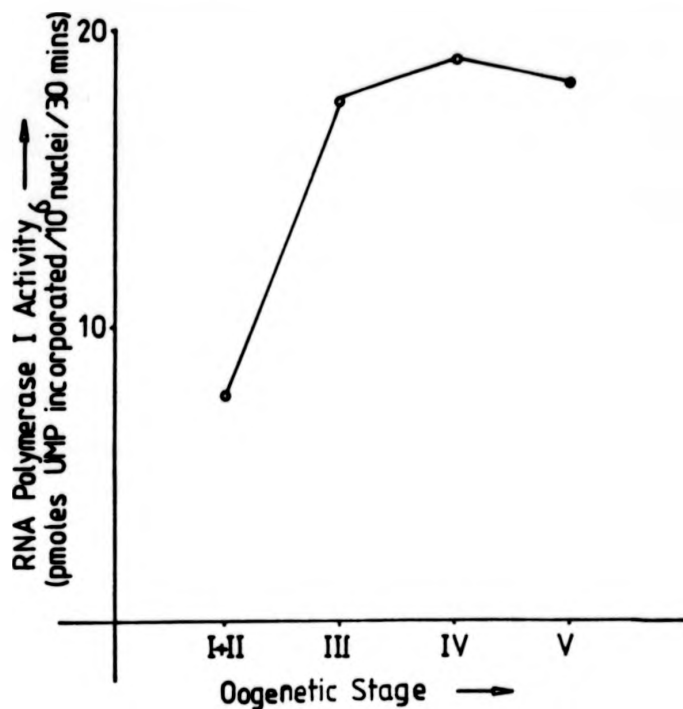


FIGURE 4.2

Effect of Oocyte Extracts from Different Stages of Oogenesis on RNA Polymerase I Transcription in Erythrocyte Nuclei.

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TABLE 4.2

RNA Polymerase I Activity in Erythrocyte Nuclei Preincubated
with a Pre-vitellogenic Oocyte Extract.

Erythrocyte nuclei plus;	pmoles UMP incorp. /10 ⁶ nuclei/30 mins.
buffer	0
egg extract	0.4
Stage I+II oocyte extract	7.6
Stage I+II oocyte extract plus buffer	3.9
Stage I+II oocyte extract plus egg extract	4.0
Total oocyte extract	16.3

Erythrocyte nuclei were preincubated in a 50 μ l assay with either buffer, egg extract, oocyte extract or a pre-vitellogenic oocyte (stage I+II) extract. Since the latter contains very little RNA polymerase I, two further assays were carried out; erythrocyte nuclei were preincubated with 10 μ l stage I+II oocyte extract and 10 μ l egg extract to provide the RNA polymerase I, or 10 μ l of buffer. All preincubations and incubations were carried out with 100 μ g/ml α -amanitin so that only RNA polymerase I transcription was seen. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the incubation.

assayed for RNA polymerase I activity, it can be seen [FIG 4.2] that all extracts elicit some activity. In the case of the vitellogenic oocyte extracts, the amount of RNA polymerase I activity is comparable to that seen in nuclei treated with an extract of whole ovary, and varies little between the smallest vitellogenic oocyte and mature oocyte extracts. Pre-vitellogenic oocyte extracts, on the other hand, although eliciting RNA polymerase I activity at a level above that seen in nuclei treated with an egg extract, do not show as high a response as when a vitellogenic oocyte extract was used. This could be a reflection of the small amount of RNA polymerase I molecules in the extract. To get round this problem, excess RNA polymerase molecules were added to the assay in the form of an egg extract. An egg extract has as much RNA polymerase activity as an oocyte extract, but does not stimulate RNA polymerase I activity in erythrocyte nuclei [FIG 3.12]. As Table 4.2 demonstrates, the addition of the extra RNA polymerases has no effect on the RNA polymerase I activity seen in erythrocyte nuclei preincubated with a pre-vitellogenic oocyte extract. The lower amount of transcription by RNA polymerase I in these nuclei, therefore, is not due to the lack of RNA polymerase I itself, but rather to the lack of the oocyte specific component necessary for ribosomal RNA gene transcription. Since it is difficult to separate out different stages of pre-vitellogenic oocytes it is not possible to say whether this component is present in small amounts in all pre-vitellogenic oocytes or only present in those oocytes approaching vitellogenesis. The amount of extract (1.20 mg) from stage V oocytes incubated with 10^6 nuclei represents the soluble protein from about 15 oocytes, whilst the same amount of protein from a pre-vitellogenic oocyte extract represents about 200 oocytes. So there is actually very little stimulatory activity in

TABLE 4.3

RNA Polymerase I Activity in Erythrocyte Nuclei Preincubated
with Extracts from Oocytes of Different Stages.

Oogenetic Stage:	pmoles UMP incorp. /10 ⁶ nuclei/30 mins. /1.2 mg protein	pmoles UMP incorp. /10 ⁶ nuclei/30 mins. /10 oocytes
I+II	7.6	0.19
III	17.6	0.86
IV	19.0	3.1
V	18.2	10.5

10 erythrocyte nuclei were preincubated with either 1.2 mg oocyte extract/100 μ l assay (left hand column) or with the soluble protein from 10 oocytes (right hand column), and with 100 μ g/ml α -amanitin. After a 60 minute preincubation the nuclei were supplemented with salts to the conditions of an incubation assay and incubated for a further 30 minutes.

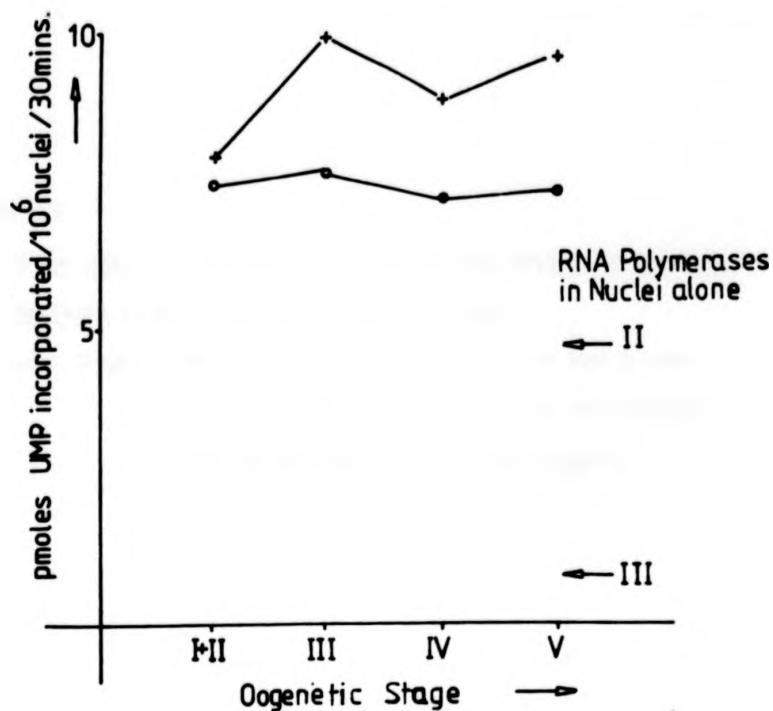


FIGURE 4.3

Effect of Different Stage Oocyte Extracts on RNA Polymerase II and III Transcription in Erythrocyte Nuclei.

The oocyte extracts described in Figure 4.1 were assayed with erythrocyte nuclei in a 50 μ l preincubation assay with and without α -amanitin present. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the incubation period. From these the pmoles UMP incorporated/10⁶ nuclei/30 minutes as a result of RNA polymerase II (o---o) and III (+---+) transcription were calculated.

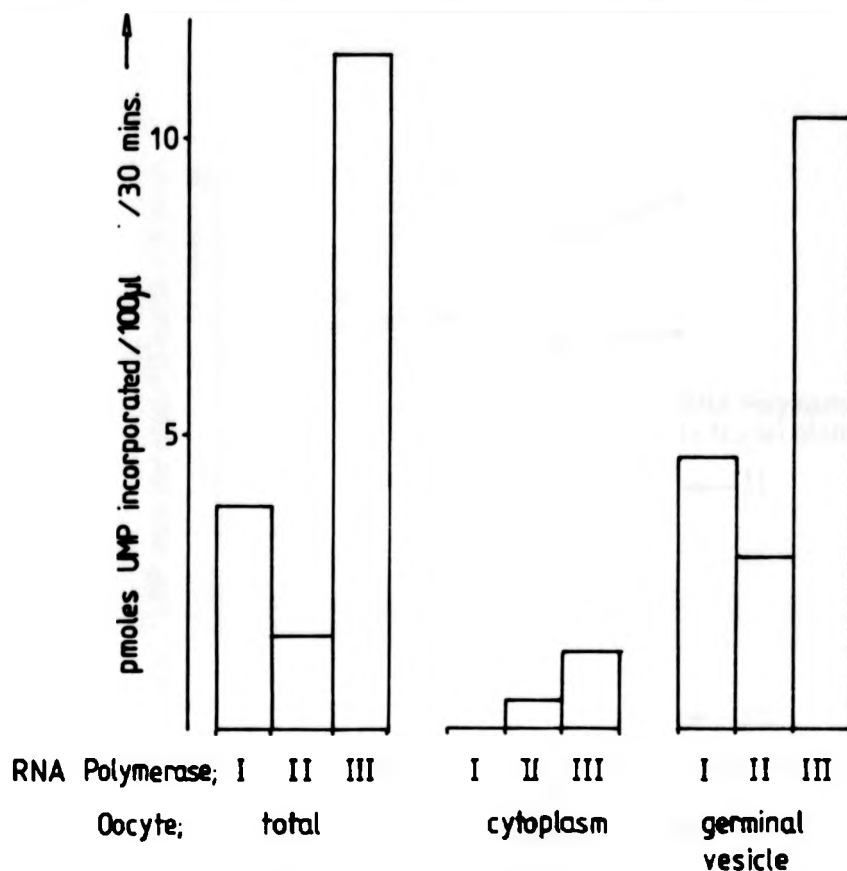


FIGURE 4.4

Relative Amounts of the Three RNA Polymerases in the Germinal Vesicle and Cytoplasm of the Oocyte.

Protein extracts from separated oocytes, enucleated oocytes and germinal vesicles were prepared in the standard way. The protein content of the oocyte and enucleated oocyte extracts were adjusted to 30 mg/ml and the final volume of the germinal vesicle extract made the same as that of the enucleated oocyte extract. These were assayed in a 50 μl assay with poly[d(A-T)] as template and α -amanitin to distinguish between the three RNA polymerases. Duplicate 10 μl samples were taken at 0 and 30 minutes of the incubation.

each pre-vitellogenic oocyte [TABLE 4.3]. If erythrocyte nuclei are preincubated with the soluble proteins from 10 oocytes [TABLE 4.3], it can be seen that there is a jump in the amount of the component/oocyte between stages III and IV. The concentration still rises though in later stages of oogenesis.

Different oocyte stage extracts have little effect on the RNA polymerase II or III activity [FIG 4.3]. The amount of transcription for both remains more or less constant. The high degree of RNA polymerase III activity seen in pre-vitellogenic oocytes relative to RNA polymerase I activity is not reflected in increased amounts of RNA polymerase III activity in erythrocyte nuclei treated with a pre-vitellogenic oocyte extract.

Location of the Ribosomal Gene Activator within the Oocyte.

This component which activates the ribosomal genes is present in vitellogenic oocytes which synthesize mostly ribosomal RNA, and is present in low levels in pre-vitellogenic oocytes where some ribosomal RNA synthesis occurs, but most of the RNAs transcribed are 4S and 5S species. The synthesis of rRNA in the oocyte itself, could also be affected by the location within the cell of regulatory molecules. The oocyte can be divided into the cytoplasm and the nucleus or germinal vesicle (GV). This is the site of transcription, not only from the 4C chromosomal DNA, but also the extrachromosomal rDNA.

In Xenopus it is easy to prepare cytoplasm-free GV's by manual techniques, leaving an intact enucleated oocyte. When the resulting extracts are assayed for RNA polymerase activity it is found [FIG 4.4] that almost all of the total transcriptional

TABLE 4.4

RNA Polymerase Activity in Erythrocyte Nuclei Preincubated With
Extracts of Oocyte GV's and Cytoplasms.

	pmoles UMP incorp. /10 ⁶ nuclei/30 mins.
column buffer	5.4
egg extract	10.3
oocyte extract	35.4
GV extract	23.9
enucleated oocyte extract	5.0
enucleated oocyte extract plus egg extract	8.5

The protein extracts from oocyte GV's and cytoplasm as described in Figure 4.4 were incubated with 5×10^5 nuclei in a standard 50 μ l preincubation assay. After 60 minutes the assays were supplemented with salts etc to give standard incubation conditions; 2x10 μ l samples were taken at 0 and 30 minutes of the subsequent incubation. Nuclei were also preincubated with 10 μ l of egg extract and 10 μ l of enucleated oocyte extract instead of the normal 20 μ l extract/assay. As controls, nuclei were preincubated with 20 μ l of column buffer, egg extract and oocyte extract.

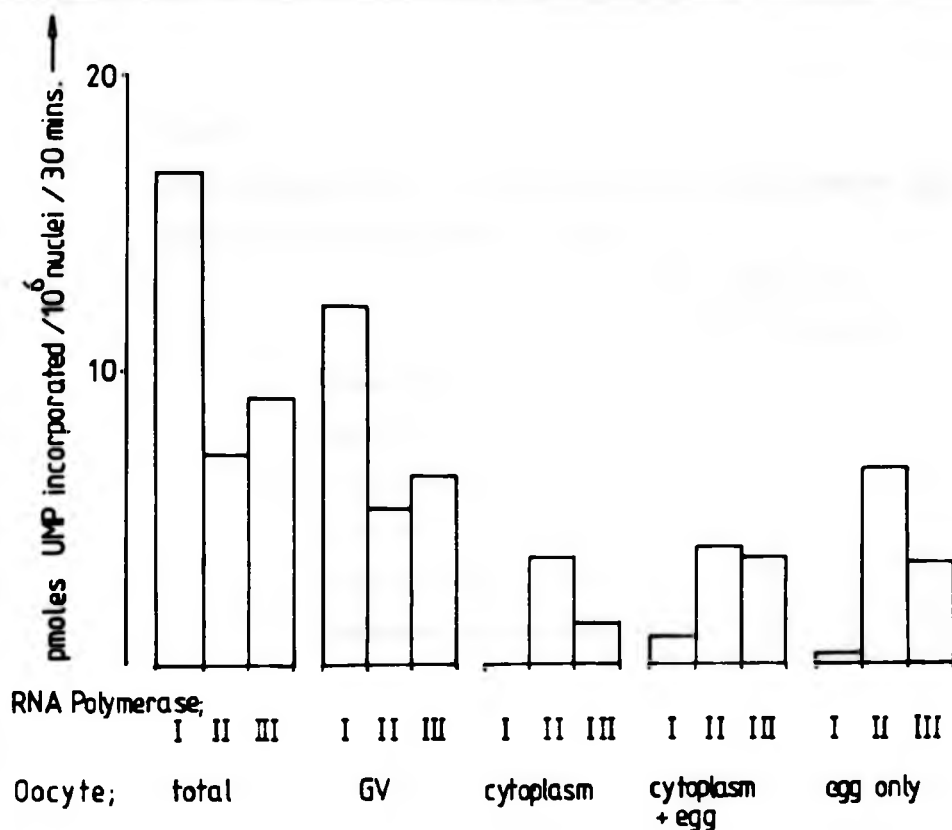


FIGURE 4.5

Relative Amounts of RNA Polymerases I, II and III Activities in Erythrocyte Nuclei Treated with Extracts from Enucleated Oocytes and Germinal Vesicles.

Erythrocyte nuclei were preincubated with the oocyte extracts described in Figure 4.4 and α -amanitin at 0, 1 and 100 μ g/ml. Nuclei were also preincubated with an egg extract, and egg extract plus the enucleated oocyte extract. After the 60 minute preincubation the assays were supplemented with salts, triphosphates and ³H-UTP, and incubated for a further 30 minutes. Duplicate 10 μ l samples were taken at 0 and 30 minutes of this incubation.

activity (91%) is located in the germinal vesicle. The little (9%) activity that is found in the cytoplasmic extract is probably due to a small amount of leakage during the preparation of the germinal vesicles. When added to erythrocyte nuclei, the enucleated oocyte extract elicits no response of increased transcriptional activity, indeed this extract is slightly inhibitory [TABLE 4.4]. This is not surprising, as detection of the oocyte component depends upon the presence of RNA polymerase I, which is present only in the nuclear extract. However, even on the addition of an egg extract to act as a source of RNA polymerases, no RNA polymerase I activity is seen above the control [TABLE 4.4; FIG 4.5]. An extract made from the purified germinal vesicles does result in increased transcription by erythrocyte nuclei [TABLE 4.4], and as can be seen, the relative levels of the three RNA polymerases active in the treated nuclei [FIG 4.5] are the same as are found in whole oocyte-treated nuclei. The oocyte component responsible for stimulating transcription by RNA polymerase on erythrocyte chromatin is therefore located exclusively in the oocyte nucleus, as might be expected since this is where all the transcription occurs.

Presence of Ribosomal Gene Activator during Early Embryogenesis.

By its presence and location during oogenesis, the activity that stimulated RNA polymerase I mirrors the transcription of the amplified ribosomal RNA genes during oocyte development. The RNA produced during oogenesis is required, at least in some cases, to last through the early development of the embryo. So whilst translation of the stored mRNA continues throughout the early

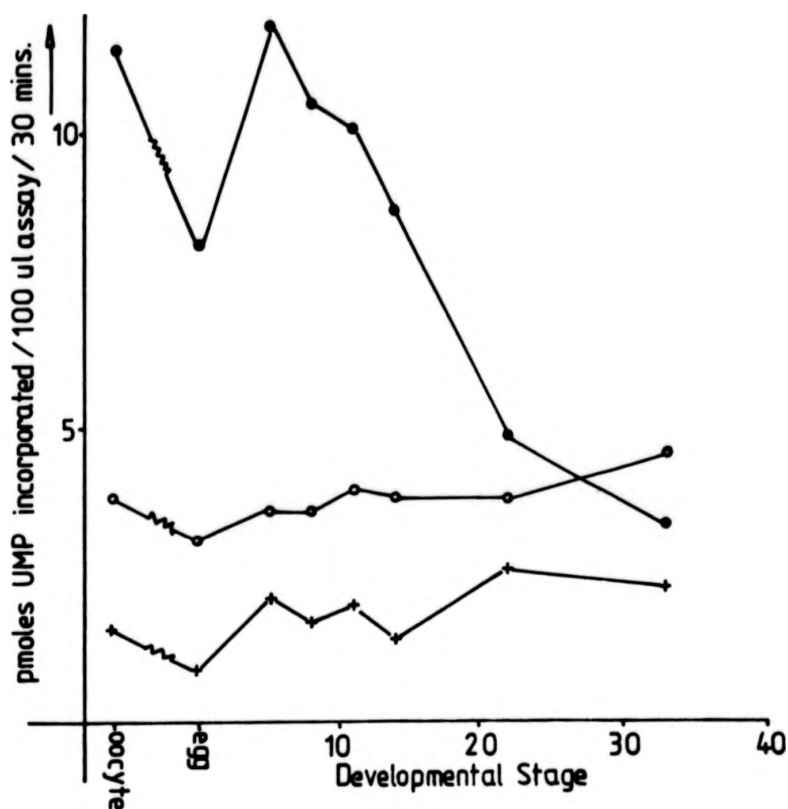


FIGURE 4.6

Levels of the Three RNA polymerases During Early Embryogenesis.

Protein extracts were prepared from oocytes, and from de-jellied eggs and embryos at various stages of development, between stages 5 and 33. These were adjusted to 30 mg/ml protein and assayed for transcriptional activity using poly[d(A-T)] as template with α -amanitin at 0, 1, or 100 μ g/ml. Duplicate 10 μ l samples were taken from the 50 μ l assays at 0 and 30 minutes of the incubation. (o---o) RNA polymerase I; (+---+) RNA polymerase II; (●---●) RNA polymerase III.

embryonic cleavages (Wasserman et al., 1982), no further RNA is transcribed until later. One explanation of this could be that transcription and rapid replication of the DNA are not mutually compatible. While it is possible to demonstrate the transcription of small RNA species (4S and 5S RNA) during mitosis (Zylber and Penman, 1971), the synthesis of larger RNA molecules is not seen in cultured cells, possibly due to the structural constraints of the condensed chromatin during mitosis. While the cells of the embryo are not mitotic for all of the time during the early stages of development, they are in S-phase for the remainder of the time. Whatever the reason for the lack of RNA synthesis seen during the early stages of embryogenesis, it is not until the early blastula stage of development that the first transcription can again be observed, and it is only when the embryo reaches late to mid blastula stage that newly synthesized ribosomal RNA (Shiokawa et al., 1981a, b) and nucleolar formation can be observed. The rRNA synthesis seen in embryos is transcribed from the chromosomal genes, the extrachromosomal copies are dispersed during oocyte maturation, are not replicated in the subsequent cell divisions and remain detectable until the early gastrula embryo (Busby and Reeder, 1982).

RNA Polymerase Levels during Embryogenesis.

Extracts made from embryos at different stages of development yield the levels of the three RNA polymerases shown in Figure 4.6, when assayed with poly[d(A-T)] as template. The relative amounts (per mg protein) of RNA polymerases I and II remain relatively stable during the development of the embryo from early cleavage to neurula. These levels are the same as found in egg and oocyte extracts. RNA polymerase III, on the

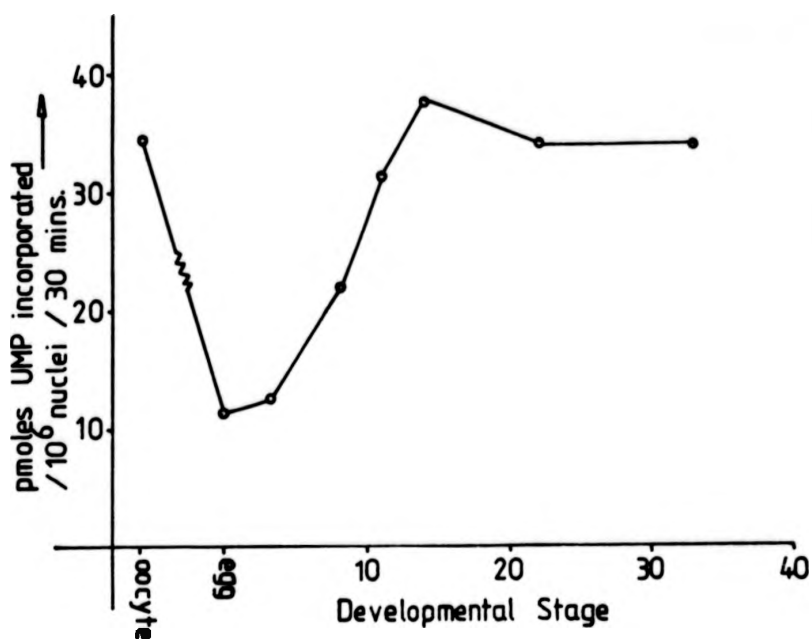


FIGURE 4.7

Effect on Erythrocyte Nuclear Transcription of Extracts from Different Stage Embryos.

The embryo extracts described in Figure 4.6 were preincubated in a 50 μ l assay with erythrocyte nuclei, supplemented with salts etc to bring the conditions to those of a standard incubation and incubated for a further 30 minutes. Duplicate 10 μ l samples were taken at 0 and 30 minutes of the incubation.

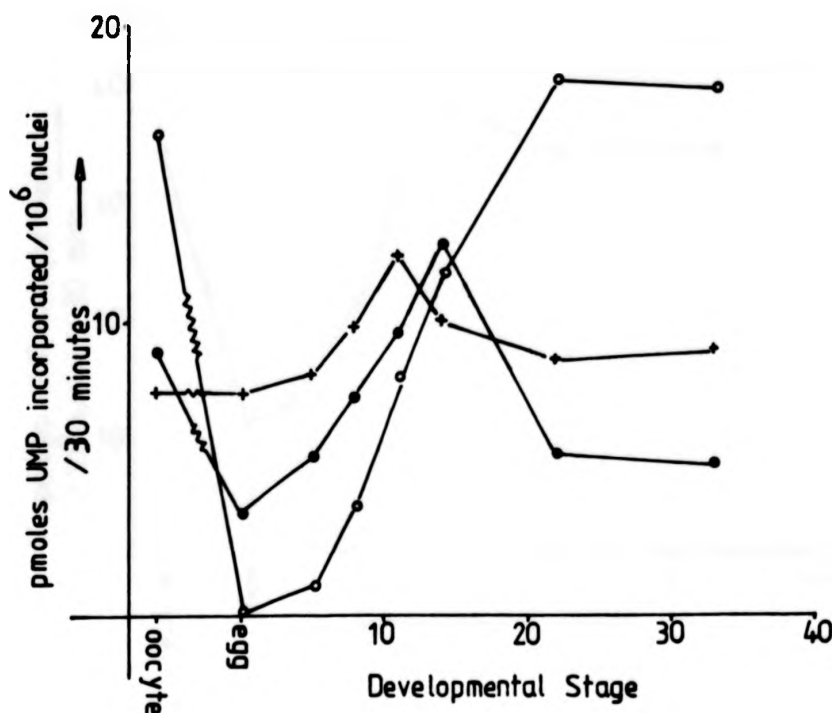


FIGURE 4.8

Relative Levels of the Three RNA Polymerases in Erythrocyte Nuclei Preincubated with Embryo Extracts.

The conditions were the same as for Figure 4.7 except that α -amanitin was included in the assay at 0, 1 and 100 μ g/ml. This gives values for the transcription of each of the RNA polymerase types; (o---o) RNA polymerase I, (+---+) RNA polymerase II, (●---●) RNA polymerase III.

other hand, exhibits an increase, which peaks at stage 5 - 11. In contrast Roeder (1974b) showed that the relative levels of the three RNA polymerases varied very little during the development of the embryo to the swimming tadpole stage, and that there are about equal quantities of the three RNA polymerases. However, in the assays I performed on these extracts, RNA polymerase III was the most abundant, at least until stage 14. These differences in amounts of the RNA polymerases and the varying amount of RNA polymerase III between different embryonic extracts is probably a reflection of the different assay conditions and of the extraction procedure which was not optimized to conserve the maximum amount of RNA polymerase activity. The drop in the activity or amount of RNA polymerase III activity after stage 11, could be brought about by the loss of chromatin associated RNA polymerase III, since the amount of tRNA synthesized per gene becomes maximal at stage 10 (Brown and Littna, 1964b; 1966).

Presence of the Ribosomal Gene Activator during Embryogenesis.

The addition of an extract made from early cleavage embryos has only a slight effect on transcription by erythrocyte nuclei compared with similar treatment with an egg extract [FIG 4.7]. Extracts from later stage embryos yielded increasing amounts of ³H-UTP incorporated into acid-insoluble material by the erythrocyte nuclei until a peak was reached with the addition of a neurula extract. A slightly lower, but constant level of RNA polymerase activity was maintained when extracts from later stage embryos were used. Using the specific RNA polymerase inhibitor, α -amanitin, the levels of all three RNA polymerases were determined for each extract on its addition to erythrocyte nuclei. As Figure 4.8 shows, RNA polymerase II activity in

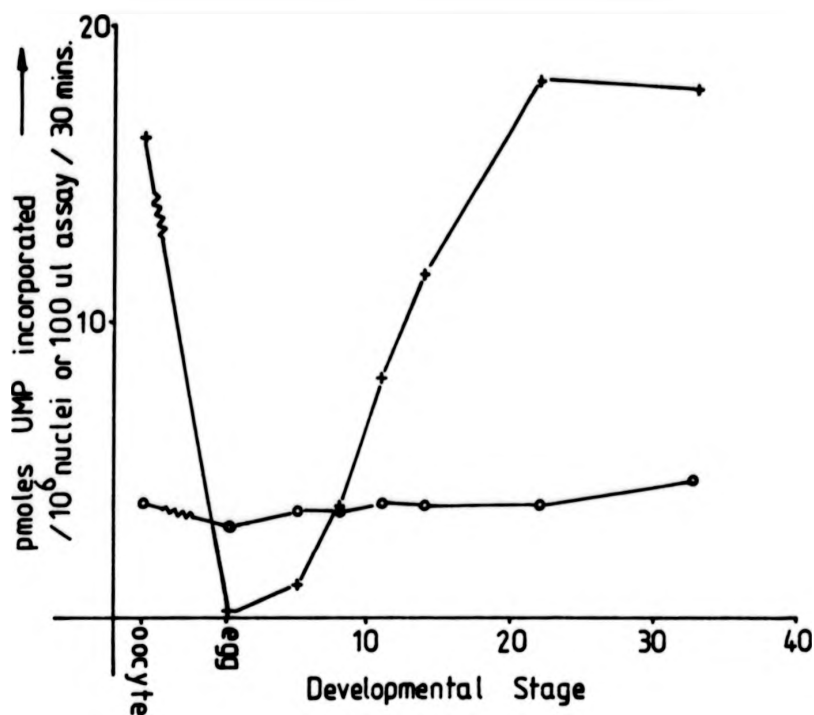


FIGURE 4.9

RNA Polymerase I Activity in Erythrocyte Nuclei Treated with Embryo Extracts.

The embryo extracts described in Figure 4.6 were assayed with 100 μ g/ml α -amanitin and either poly[d(A-T)] (o---o) or erythrocyte nuclei (x---x), such that only transcription by RNA polymerase I was possible. Experimental details as in Figures 4.7 and 4.8. The data are expressed as pmoles UMP incorporated/ 10^6 nuclei for the erythrocyte nuclear assay, or pmoles UMP incorporated/100 μ l assay for when poly[d(A-T)] was used as template.

treated nuclei varies little between oocyte, egg and early cleavage extracts, it shows a slight increase in activity in blastula and gastrula extracts and then declines slowly. A slight peak of activity, albeit only 1.2 fold higher than in nuclei treated with egg extract, is seen in nuclei treated with a gastrula extract. RNA polymerase III activity follows a similar pattern, but delayed slightly, such that a peak of activity is seen in neurula-treated nuclei, at 2.9 fold above the level in egg-treated nuclei. The activity thereafter drops to a level similar to that seen in nuclei preincubated with an egg or an early cleavage extract. The peak in RNA polymerase III transcription in the extract-treated nuclei is not related to the levels of RNA polymerase III molecules seen in the extracts themselves, since these are at their highest at the earlier stages of development and are declining when the RNA polymerase III activity in embryo-treated nuclei is increasing [FIG 4.6]. The activity of RNA polymerase I in these embryo-treated nuclei also varies little between egg and early cleavage embryo extracts. In both cases it is low, at a level well below that seen in oocyte-treated nuclei. After this stage, the activity increases until in nuclei treated by a neurula extract, about a third of the transcription is due to RNA polymerase I activity. This activity continues to increase until stage 22 when it reaches a level equivalent to that seen in oocyte-treated nuclei and accounts for about 56% of the total RNA synthesis. As Figure 4.9 demonstrates, although the activity of RNA polymerase I in the erythrocyte nuclei varies between different developmental stages, mimicking endogenous embryonic nuclei, the actual amount of RNA polymerase I activity in whole embryos remains constant.

So it could be proposed that the molecule(s) present in the

oocyte that are responsible for bringing about ribosomal gene transcription in treated erythrocyte nuclei is absent in the egg and early cleavage stage embryos. The same molecule, or one that achieves the same effect would appear later on during embryonic development. Its absence in the egg would account for the low level of rRNA synthesis seen at this stage. It would then build up slowly through the early stages of development from blastula onwards so that by stage 22 it is at its maximum concentration. This build up could occur in either of two extreme ways, or as a mixture of the two, to account for the intermediate values between blastula and neurula stage embryos. All cells might gradually accumulate the component during development, either by synthesizing these molecules anew or by reactivating a store of them, for example by phosphorylation, so that all cells have an identical amount of this component; or, some cells of the embryo might contain high, or maximal levels of the component and others contain very low amounts, or none at all. During development therefore, more cells would switch from containing no active ribosomal component to containing a high level. In the earlier stages a disparity might be expected in the ribosomal RNA synthesizing ability of different cells, which would disappear as development progressed. It has been shown (Woodland and Gurdon, 1968; Misumi et al, 1980) that there are indeed at least two distinct cell populations in neurulae with respect to both their ribosomal RNA synthesis and their eventual differentiation. Endoderm cells from these embryos synthesize little rRNA, whilst the remaining embryonic cells do so. This disparity between the ability of these cells to synthesize rRNA is not seen in later stage embryos (Woodland and Gurdon, 1968; Misumi et al, 1980). Although the first explanation cannot be completely ruled out, it would seem likely that the second possibility could

explain in part the gradual accumulation of the stimulatory component during embryonic development.

TABLE 5.1

Effect of Increasing Centrifuge Speed on Oocyte and Egg
Extracts.

Extract	RCF(xg)	pmoles UMP incorp. /10 ⁶ nuclei/30 mins.	Stimulation
buffer	--	5.9	1
egg	8,000	11.9	2.02
egg	20,000	11.6	1.97
egg	100,000	10.7	1.81
oocyte	8,000	36.1	6.12
oocyte	20,000	35.6	6.03
oocyte	100,000	38.7	6.56

Dejellied eggs or oocytes were homogenized in WH buffer and spun at 8,000g, 20,000g or 100,000g. The resulting soluble protein extracts were adjusted to 30 mg protein/ml and tested in a normal 50 μ l preincubation plus incubation assay for total transcriptional activity.

CHAPTER V; PURIFICATION BY COLUMN CHROMATOGRAPHY OF THE COMPONENT OF OOCYTES WHICH STIMULATES RNA POLYMERASE I.

All of the extracts studied in the preceding chapters are very crude preparations. They are mixtures of all the proteins, nucleic acids and constitutive ions that are left when the lipids and insoluble proteins are removed. This is achieved by a series of centrifugation steps. These increasing centrifuge speeds do not result in any loss of activity [TABLE 5.1]; even a 100,000g spin, that removes most of the ribosomes in the sample, has little effect on its stimulation of RNA synthesis. Neither does it cause an egg extract to show this activity, as might be expected if a sedimentable inhibitor was causing the different effects of oocyte and egg extracts. It will be noticed, that the specific activity increases during the centrifugation process. This is because a number of non-stimulating proteins are lost at each step. If the extracts are standardized according to the number of oocytes in the starting material, rather than according to the mass of protein, this effect is not seen. The observation that oocyte extracts prepared using a 100,000g spin can still stimulate RNA polymerase I activity in erythrocyte nuclei means that the molecule(s) responsible is not tightly bound to a subcellular structure that remains intact during the homogenization and subsequent centrifuge steps, and sediments at the same speed or faster than ribosomes.

Sephadex G100 Column Chromatography of Oocyte Extracts.

From the study of crude extracts, it is not possible to say whether the relevant oocyte component is a single molecule, or several working in concert. Nor is it possible to say whether the

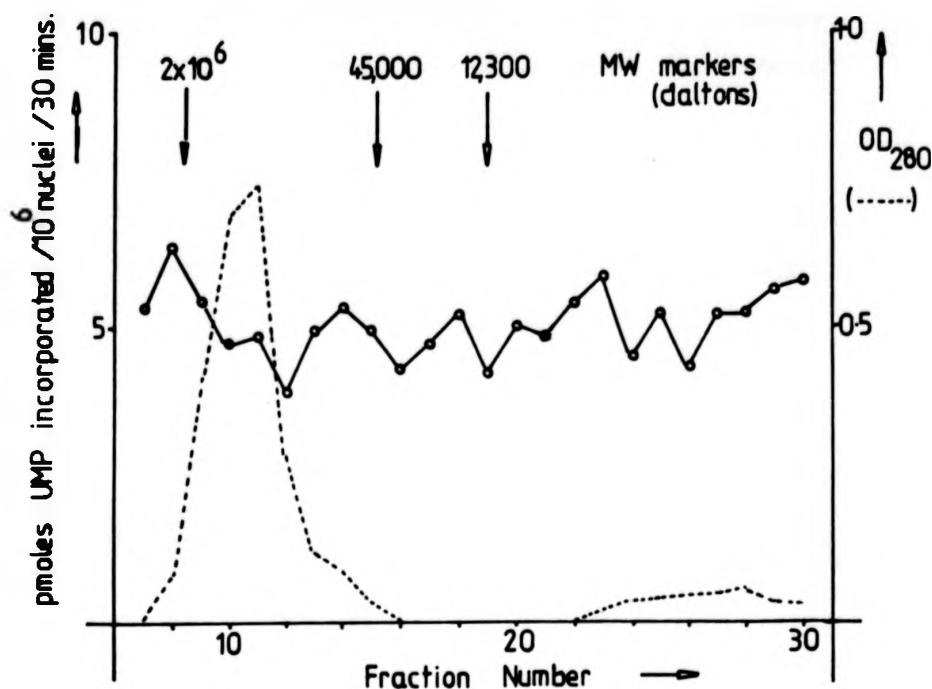


FIGURE 5.1

Fractionation by Sephadex G100 of an Oocyte Extract.

A 200 μ l aliquot of oocyte extract was loaded onto a G100 column (2 cm diameter by 23 cm), the 30 drop fractions were collected and stored at -20°C . These were assayed with erythrocyte nuclei, but no additional polymerase or egg extract, in 50 μ l preincubation and incubations (20 μ l column fraction, 5×10^5 nuclei), two 10 μ l samples were taken at 0 and 30 minutes of the incubation.

activity seen in nuclei treated with a neurula extract is brought about by the same or different molecules from those in the oocyte extract. I have attempted to explore these possibilities by fractionating neurula and oocyte extracts on Sephadex and DEAE cellulose columns. Of course, two dissimilar proteins might still co-elute after these chromatographic steps, and the existence of a single stimulatory column fraction does not mean there is only a single protein species responsible.

The OD profile of an oocyte extract after passage through a Sephadex G100 column is shown in Figure 5.1. The first peak contains the largest of the protein molecules and the high molecular weight nucleic acids. The second, smaller, peak contains the smallest molecules, eg free triphosphates and mineral ions, whose passage have been most impeded through the column due to their ^{small} size. The column was calibrated with molecular weight marker proteins.

When the column fractions were assayed with erythrocyte nuclei in a standard preincubation assay, no increase in transcription was seen on the addition of any of these fractions [FIG 5.1]. This means that even the endogenous RNA polymerase II was not affected by these fractions. In the case of RNA polymerase I an effect could hardly be expected, since the nuclei contain no RNA polymerase I [FIG 3.8] and none was present and active in the column fractions (see later). This deficiency was rectified by adding egg extract as the polymerase source; this is possible because the egg extract itself does not affect RNA polymerase I transcription of the ribosomal genes [FIG 3.12]. The approach is analogous to the studies of Engelke et al (1980) on 5S genes. Under these assay conditions, a peak of stimulated

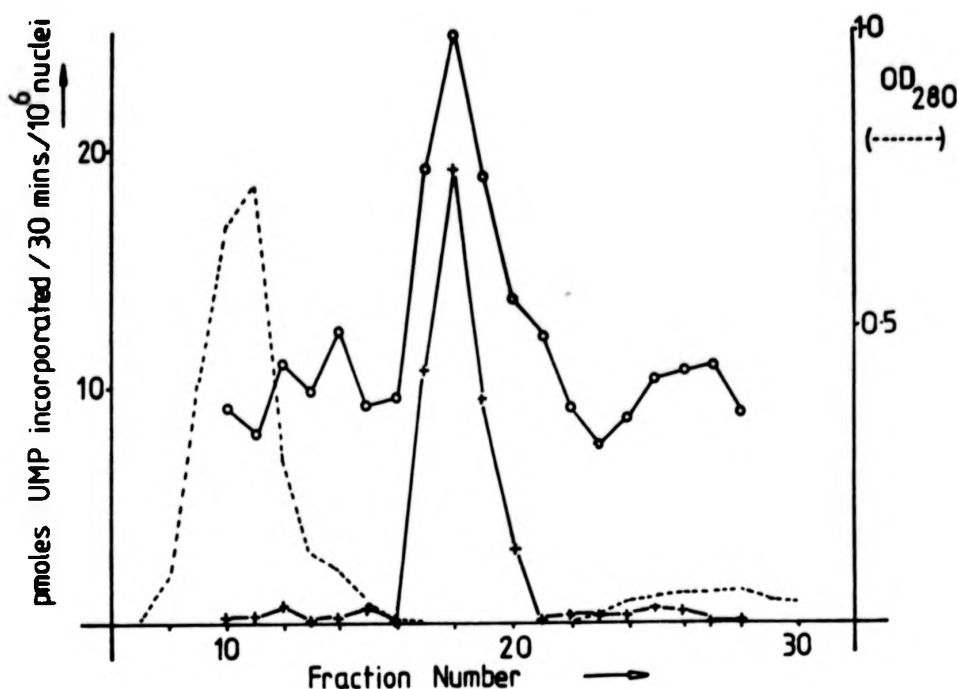


FIGURE 5.2

Oocyte G100 Column Assay with Erythrocyte Nuclei and Egg Extract.

The column fractions described in Figure 5.1 were assayed using an egg extract as the RNA polymerase source such that a 50 μ l preincubation contained 10 μ l column fraction, 10 μ l egg extract and 5×10^5 nuclei (o---o). These were supplemented with salts etc to a final volume of 63 μ l after 60 minutes, and duplicate 10 μ l samples taken at 0 and 30 minutes of the subsequent incubation. A parallel column assay was also carried out, but with 100 μ g/ml α -amanitin present to prevent all but RNA polymerase I activity (+---+).

transcriptional activity is seen [FIG 5.2]. It elutes slightly before cytochrome c and has an estimated molecular weight of 19500 - 21000 daltons. The single peak of activity is constant between different columns and different oocyte extracts, the active component(s) of this peak was designated Fr-18.

In order to determine which of the three RNA polymerases present in the egg extract is responsible for this increase in transcription, the column assay was repeated using α -amanitin. As previously mentioned, Fr-18 has no effect on transcription by the endogenous RNA polymerases in erythrocyte nuclei. Figure 5.2 demonstrates that the stimulation of transcription brought about by fractions 17-19 is resistant to high (100 μ g/ml) levels of α -amanitin. The transcription is therefore a result of RNA polymerase I activity. No further peaks of transcriptional activity can be seen on adding α -amanitin. Small peaks might have been masked by the amount of variation seen in fractions away from the main peak; indeed the amount of variation seen about the control value is decreased by adding the inhibitor.

The amount of stimulation observed in nuclei treated with Fr-18 is comparable with nuclei treated with oocyte extract, despite a 20-fold dilution by the column procedure. This increase in activity by dilution may be produced by loss of some inhibitor of transcription, or the loss of any RNases that are present in the oocyte extract.

The Sephadex results show that the stimulation of RNA polymerase I transcription in nuclei plus egg extract is produced either by a single protein species or by multiple protein species of the same molecular weight. Although the stimulation seen in

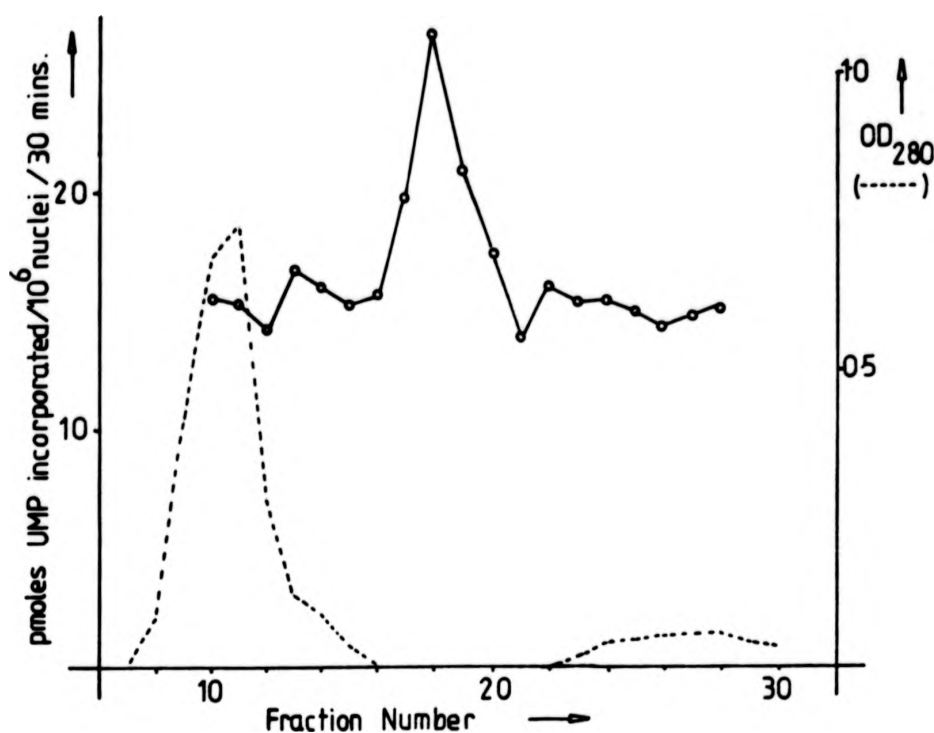


FIGURE 5.3

Oocyte G100 Column Assay with Erythrocyte Nuclei, Egg Extract and Fr-18.

The column fractions described in Figure 5.1 were assayed using erythrocyte nuclei (5×10^5 nuclei/assay), 7 μ l egg extract, 7 μ l Fr-18 and 7 μ l column fraction in a final 50 μ l volume. These were preincubated for 60 minutes, supplemented with salts etc and incubated for a further 30 minutes. Samples were taken at 0 and 30 minutes of the incubation.

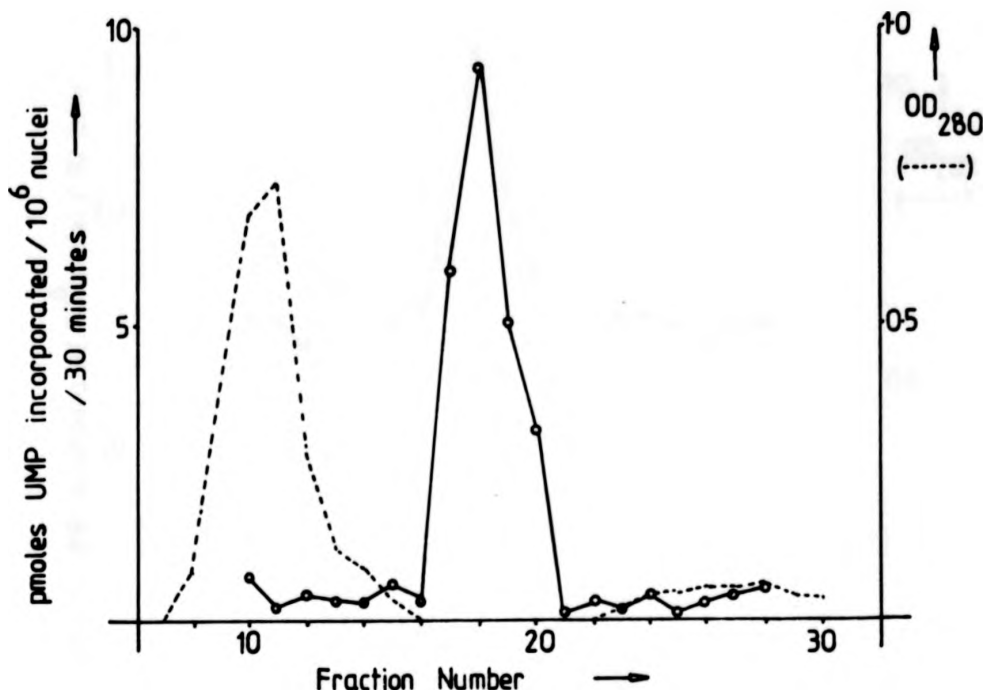


FIGURE 5.4

Oocyte G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.

10 μ l aliquots of the oocyte G100 column fractions were preincubated with 5×10^5 erythrocyte nuclei, 100 μ g/ml α -amanitin plus semipurified RNA polymerases in a final volume of 50 μ l. After 60 minutes the assays were supplemented with salts etc and incubated for 30 minutes. 2x10 μ l samples were taken at 0 and 30 minutes of the incubation to determine the RNA polymerase I activity.

nuclei treated with oocyte extract is more than accounted for by this peak, it is still possible that there is a second oocyte component which depends not only on the presence of RNA polymerase I, but also on the presence of Fr-18. Therefore, the column assay was repeated, this time having both egg extract and fraction 18 within the assay mix [FIG 5.3]. No other regions within the column yielded any increase in activity, the only peak in transcription observed being that around fraction 18. It appears, therefore, that only Fr-18 is required for RNA polymerase I transcription of erythrocyte rRNA genes, or, if something else is necessary, it is present in the egg extract.

In order to test if there was anything in the egg extract that was necessary to see a peak at fractions 17-19 apart from the RNA polymerases, the egg extract was substituted by a crude RNA polymerase preparation. The resulting assay [FIG 5.4] showed a similar peak of activity in fractions 17-19 to that obtained with an egg extract. It is resistant to 100 $\mu\text{g/ml}$ α -amanitin indicating that it is RNA polymerase I that is required. The RNA polymerase preparation was crude, being only a 10-40% $(\text{NH}_4)_2\text{SO}_4$ precipitate of an oocyte extract, so it is possible that it contains another essential substance, that is also present in the egg extracts.

There remains the possibility that Fr-18 does not have a specific effect on the transcription of the ribosomal genes by RNA polymerase I. (That the ribosomal genes are involved will be shown in Chapters VI and VII). Two possibilities for this non-specific function are that the fraction contains further RNA polymerase and the presence of a DNA nicking enzyme. These can be ruled out. Although excess RNA polymerase I has no effect on

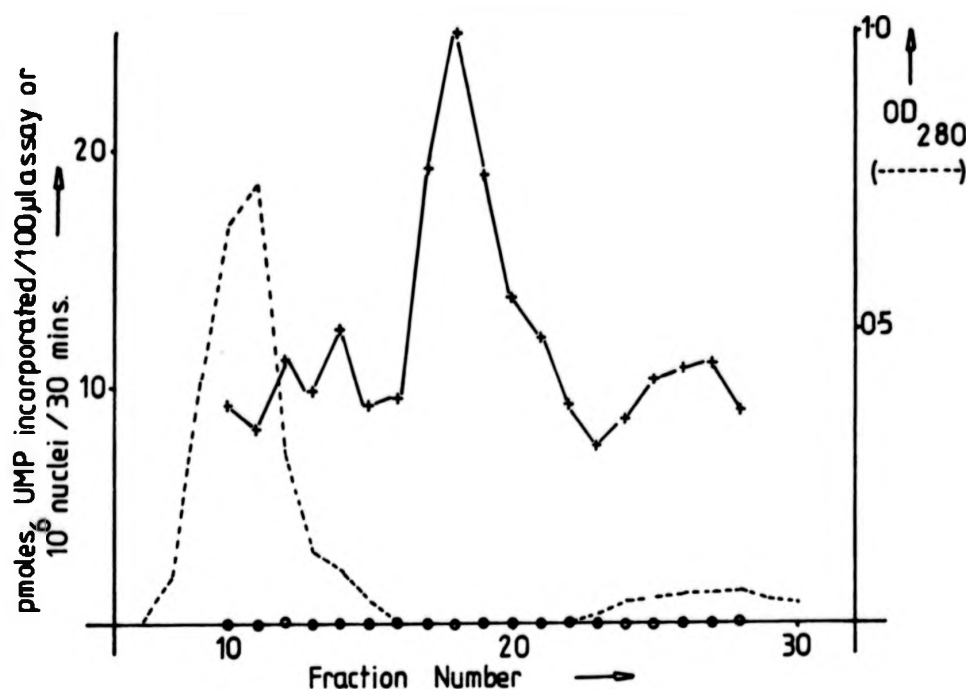


FIGURE 5.5

Oocyte G100 Column Assay with poly[d(A-T)].

The oocyte G100 column fractions were tested for RNA polymerase activity by incubating 20 μ l aliquots of the fractions with 2 μ g poly[d(A-T)] in 50 μ l assays for 30 minutes (o---o). A parallel series of assays were carried out on 10 μ l aliquots of the column fractions with 10 μ l egg extract and 5×10^5 nuclei (+---+). These were preincubated for 60 minutes, supplemented and incubated for a further 30 minutes. In both cases 2x10 μ l samples were taken at 0 and 30 minutes of the incubation.

erythrocyte nuclei and the molecular weight of an RNA polymerase would ensure that it would pass through the column in the void fraction, it could be argued that Fr-18 contains some kind of RNA polymerase activity. However, as Figure 5.5 shows, when the column fractions are assayed using poly[d(A-T)] as template no activity is seen in any of the fractions, including fraction 18. It is a little surprising that the early fractions contain no RNA polymerase activity. However the fractions were stored at -20°C without glycerol or any other stabilizing chemical, which together with the relatively small amount of RNA polymerase activity in the original extract and the 20-fold dilution may explain the lack of subsequent RNA polymerase activity in the column fractions.

It is known that both RNA polymerases I and II preferentially initiate transcription at single stranded sites within the double stranded DNA template (Seifart, 1971). Thus if fraction 18 contained a nicking enzyme, its stimulatory activity could be a reflection of its ability to produce unspecific breaks in the erythrocyte DNA. This does not, however, explain why only stimulation of RNA polymerase I is seen, since RNA polymerase II is also present in the egg extract. The column fractions were again assayed, but this time using the supercoiled plasmid pXLr14 in place of the nuclei. In this way, by incubating the plasmid in identical salt conditions to the nuclear assay followed by isolating the DNA and running it out on an agarose gel, it was possible to detect nicking by the disappearance of supercoils. It is known that an oocyte extract contains a variety of enzymes, including nicking-closing enzymes, that would cause a supercoiled plasmid to become relaxed. None of these are associated with fractions 17-19; rather they are found at fractions 10-13 [FIG

... v.
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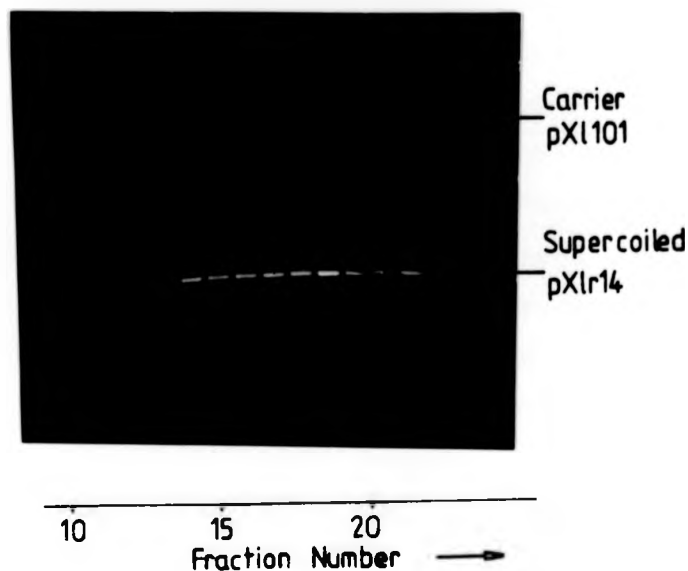


FIGURE 5.6

DNA Nicking Activity in the Oocyte G100 Column Fractions.

0.3 μ g samples of plasmid pXlr14 were incubated in a standard incubation mix with 10 μ l each column fraction at 25°C in a series of 25 μ l assays. After 90 minutes 0.1 μ g pX1101 was added as carrier, the samples were extracted once with phenol-chloroform (1:1), once with chloroform, made 0.3 M sodium acetate and ethanol precipitated. The precipitated DNA was taken up in sample buffer and run on a 0.8% agarose gel overnight. The gel was stained with ethidium bromide and photographed under UV light.

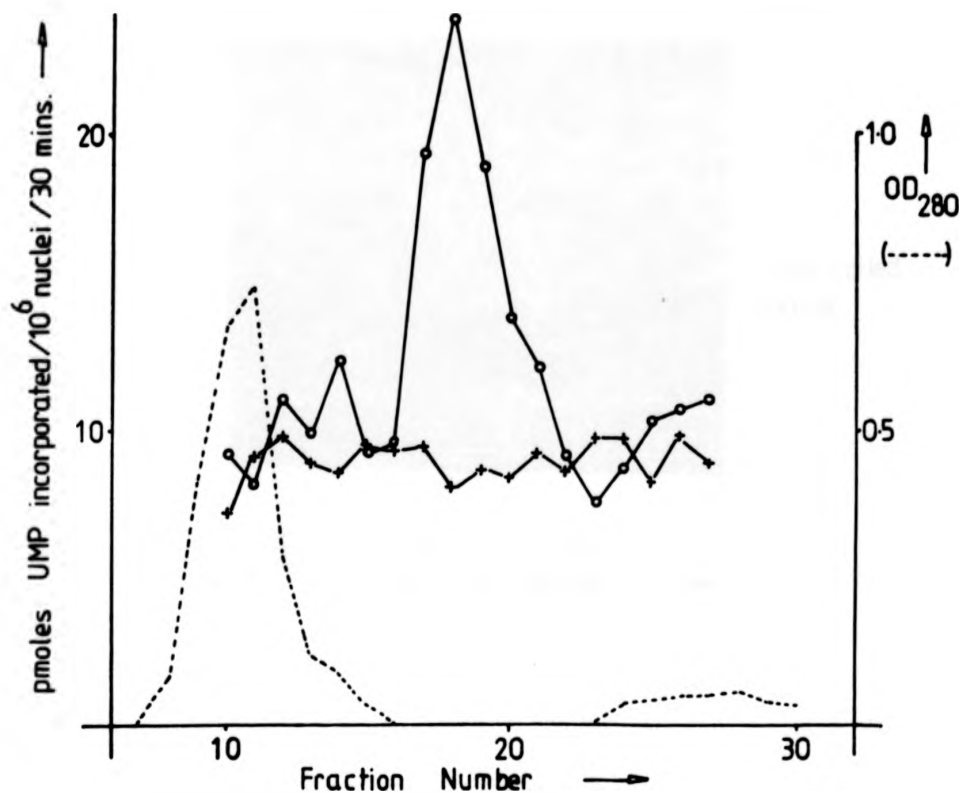


FIGURE 5.7

Heat Inactivation of Fr-18.

50 μ l samples of oocyte G100 column fractions 10-27 were boiled for 10 minutes and cooled before 10 μ l of each was added to a standard 50 μ l column assay (+---+; 5×10^5 nuclei, 10 μ l egg extract). A parallel assay was carried out using unboiled column fractions (o---o). The assays were preincubated for 60 minutes, supplemented and incubated for a further 30 minutes. Duplicate 10 μ l samples were taken at the beginning and end of the incubation.

TABLE 5.2

Trypsin Inactivation of Fr-18.

Preincubation Ingredients;	pmoles UMP incorp. /10 ⁶ nuclei/30 mins.
Nuclei + buffer	7.6
oocyte extract	23.7
boiled oocyte extract	7.9
trypsin x oocyte extract	7.3
Fr-18	24.7
trypsin x Fr-18	7.9
trypsin-trypsin inhibitor x oocyte extract	19.5
trypsin-trypsin inhibitor x Fr-18	22.7

100 μ l samples of an oocyte extract and Fr-18 were incubated with trypsin or trypsin plus trypsin inhibitor for 30 minutes. Trypsin inhibitor was added to the trypsin digestions at the end of this time. A 50 μ l sample of oocyte extract was also boiled for 10 minutes. These samples (10 μ l) were preincubated with 5×10^5 nuclei and 10 μ l egg extract for 60 minutes before being supplemented with salts etc and incubated for a further 30 minutes. As controls, nuclei were preincubated with 10 μ l of column buffer or 10 μ l oocyte extract and 10 μ l egg extract. Protein samples were incubated with trypsin at 500 μ g/ml for 15 minutes at 37°C prior to the addition of trypsin inhibitor (500 μ g/ml) and assay.

5.6]. This does not rule out a nicking enzyme being a functional component in the stimulation by Fr-18, since these would be present in the egg extract, and possibly in the RNA polymerase preparation as well. However, since neither of these on their own have any effect on transcription of erythrocyte chromatin by RNA polymerase I, some other component of the oocyte must still be necessary to stimulate RNA polymerase I.

It can be shown that the active component of fraction 18 is, or includes, a protein by demonstrating its sensitivity to trypsin treatment and boiling. As Figure 5.7 shows, boiling the column fractions results in the loss of the peak at fractions 17-19. Other components of the oocyte extract are also heat degradable [TABLE 5.2], eg RNA polymerases, and extra have to be supplied, in this case as egg extract. After trypsin treatment both the oocyte extract and Fr-18 lose their ability to stimulate RNA polymerase I activity [TABLE 5.2]. Egg extract was also added to these assays as the RNA polymerase I source. If trypsin inhibitor is added at the same time as the trypsin, instead of before the transcription assay, the oocyte extract and Fr-18 do not lose any potency. Therefore it is likely that that whatever is bringing about this effect is a protein.

Sephadex G100 Column Chromatography of Germinal Vesicle and Enucleated Oocyte Extracts.

It has been demonstrated previously (Chapter IV) that the protein responsible for bringing about transcription by RNA polymerase I is located within the germinal vesicle of the oocyte. An enucleated oocyte extract has no effect on erythrocyte nuclear transcription, even when excess RNA polymerases are

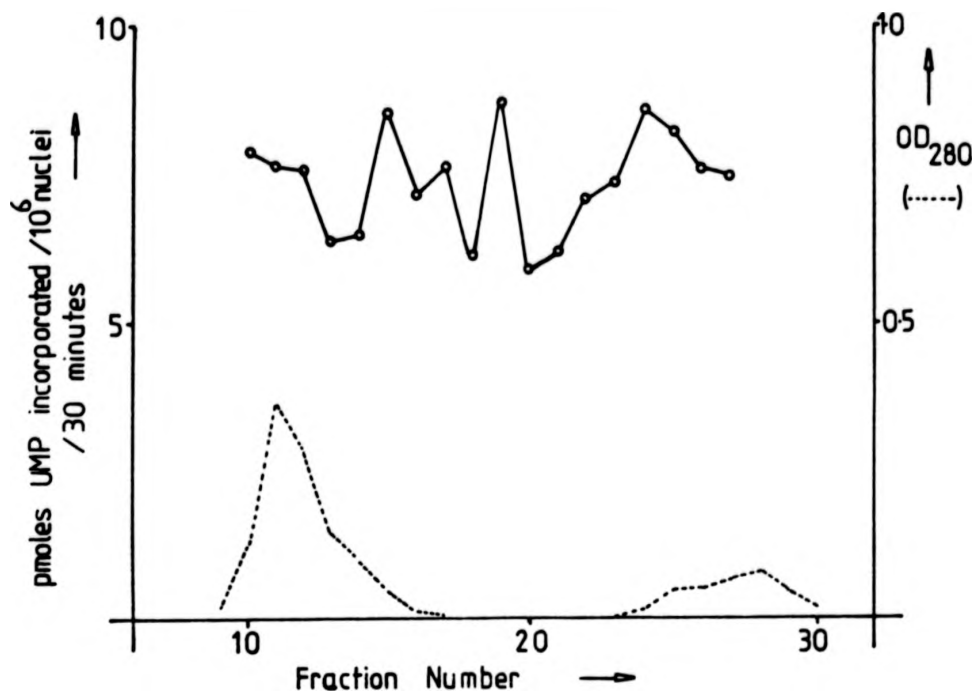


FIGURE 5.8

Fractionation by Sephadex G100 of an Enucleated Oocyte Extract.

150 μ l of an enucleated oocyte extract was loaded onto a Sephadex G100 column (2 cm diameter x 23 cm) and the resulting 30 drop fractions collected and stored at -20°C . 10 μ l aliquots of these fractions were added to 50 μ l column assays (5×10^5 nuclei, 10 μ l egg extract), preincubated for 60 minutes, supplemented and incubated for 30 minutes. 2x10 μ l samples were taken at 0 and 30 minutes of the incubation.

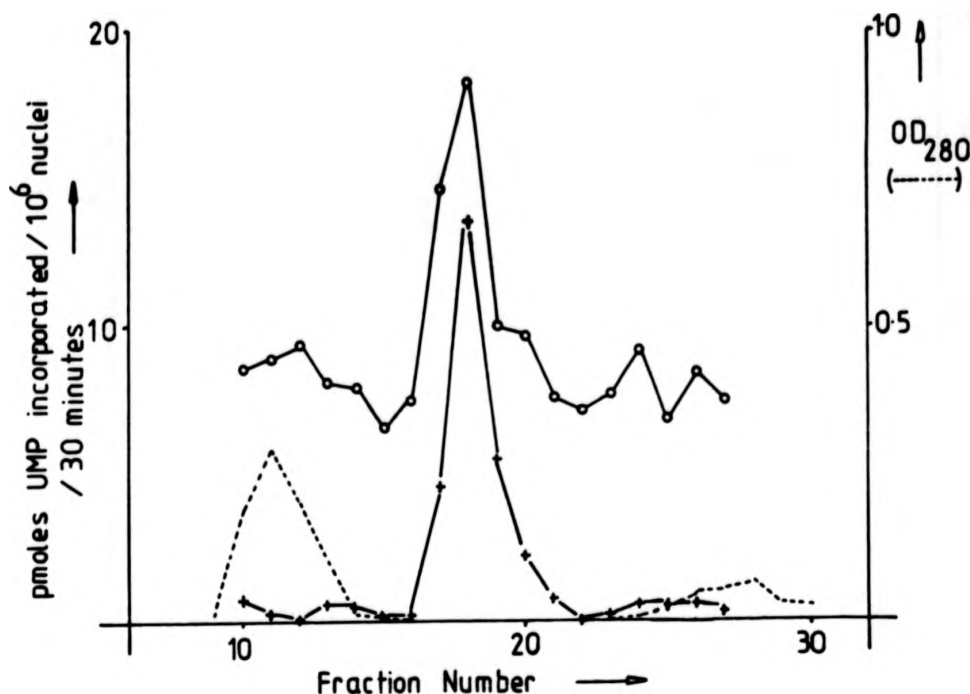


FIGURE 5.9

Fractionation by Sephadex G100 of an Oocyte Germinal Vesicle Extract.

100 μ l of an oocyte germinal vesicle extract was loaded onto a Sephadex G100 column (2 cm diameter x 23 cm), 30 drop fractions were collected and stored at -20°C . 10 μ l aliquots of fractions 10 to 27 were preincubated with 5×10^5 nuclei and 10 μ l egg extract with (+---+) or without (o---o) 100 $\mu\text{g/ml}$ α -amanitin. After a 60 minute preincubation period the assays were supplemented and incubated for 30 minutes.

present. Sephadex G100 columns were run of both oocyte components - the germinal vesicle and the remaining cytoplasm. When assayed, using an egg extract as the RNA polymerase source, the enucleated oocyte column [FIG 5.8] yielded no peaks of higher transcriptional activity. None of the resulting column fractions had any effect on transcription. The germinal vesicle column gave one peak of heightened transcriptional activity - fractions 17-19 [FIG 5.9]. This corresponds in its location to the peak seen when an extract of whole oocytes is fractionated. The activity has an estimated molecular weight of 19750 to 21250 daltons. The level of transcription seen in this peak is somewhat lower, which is to be expected since only 50 germinal vesicles were loaded onto this column, as compared with 100 whole oocytes that were normally used. As expected, α -amanitin shows that the increased transcription in the nuclei incubated with fractions 17-19 is a result of RNA polymerase I [FIG 5.9]. Therefore the active component found in the oocyte extract G100 column fractions is located in the oocyte nucleus.

Sephadex G100 Column Chromatography of Egg Extract.

In terms of transcription by RNA polymerase I, an egg extract elicits no response when incubated with erythrocyte nuclei, despite containing that RNA polymerase. This does not necessarily mean that the stimulatory protein found in the oocyte extracts is absent from the egg extract, although this is the simplest explanation. It could be chemically modified eg glycosylated or phosphorylated, or perhaps be tightly bound to or in equimolar amounts with an inhibitor. An egg extract was run through a Sephadex G100 column and the resulting fractions assayed in the standard way. As Figure 5.10 shows, there is no

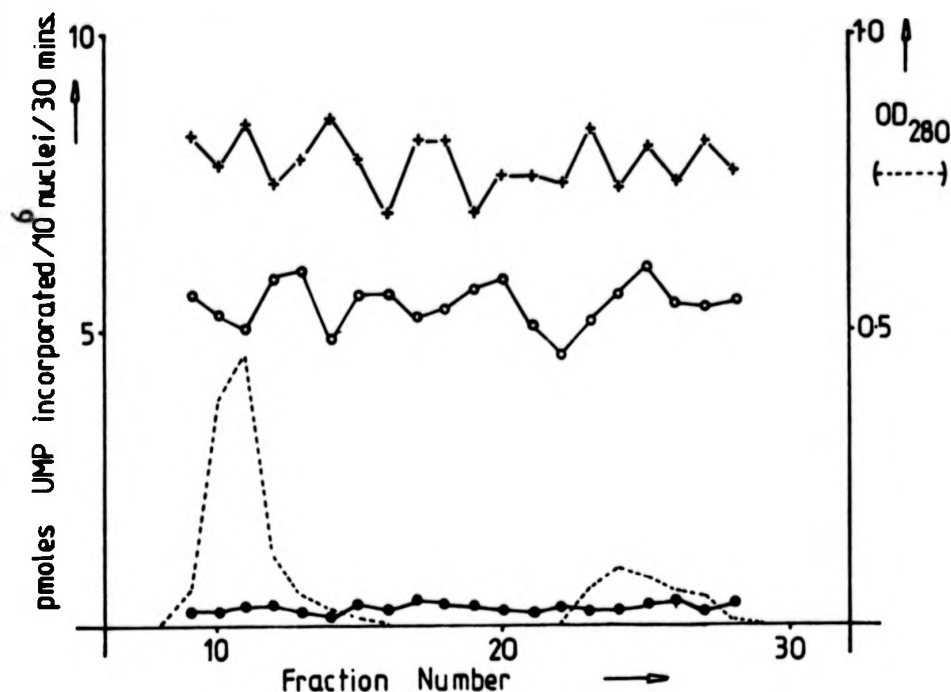


FIGURE 5.10

Fractionation by Sephadex G100 of an Egg Extract.

A 200 µl aliquot of egg extract was loaded onto a G100 (2 cm diameter x 23 cm) and the resulting 30 drop fractions collected and stored at -20°C. Three column assays using these fractions were carried out, 5×10^5 nuclei were preincubated with 20 µl column fraction (o---o), 10 µl column fraction plus 10 µl egg extract (+---+) or 10 µl column fraction, 10 µl egg extract and 100 µg/ml α-amanitin (●---●). These were preincubated for 60 minutes, supplemented and incubated for 30 minutes.

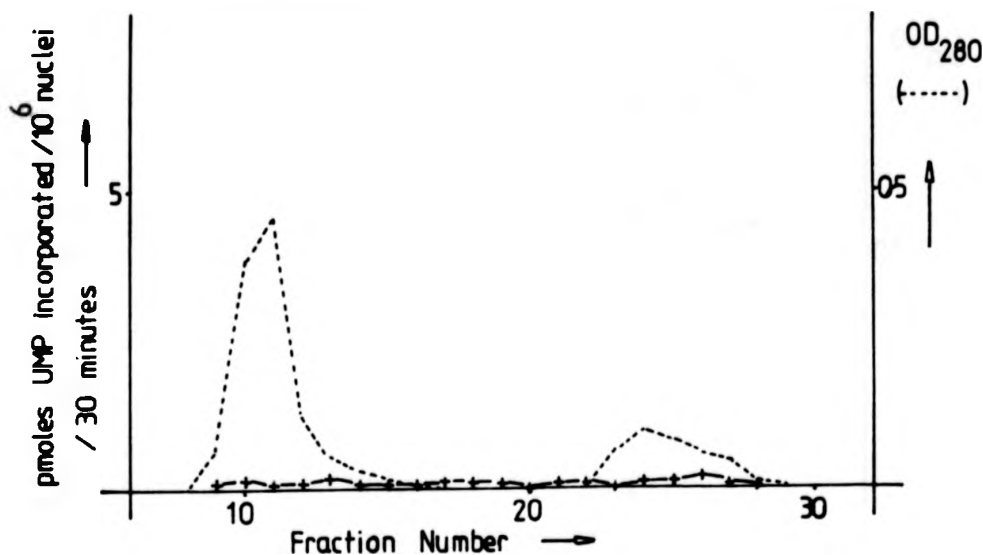


FIGURE 5.11

Egg G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.

15 μ l of egg G100 column fractions 9 to 28 were added to 5×10^5 nuclei and semipurified RNA polymerase with α -amanitin at 100 μ g/ml in a final volume of 50 μ l, such that only RNA polymerase I transcription was possible. These were preincubated for 60 minutes, supplemented and incubated for a further 30 minutes. Samples ($2 \times 10 \mu$ l) were taken at 0 and 30 minutes of the incubation period.

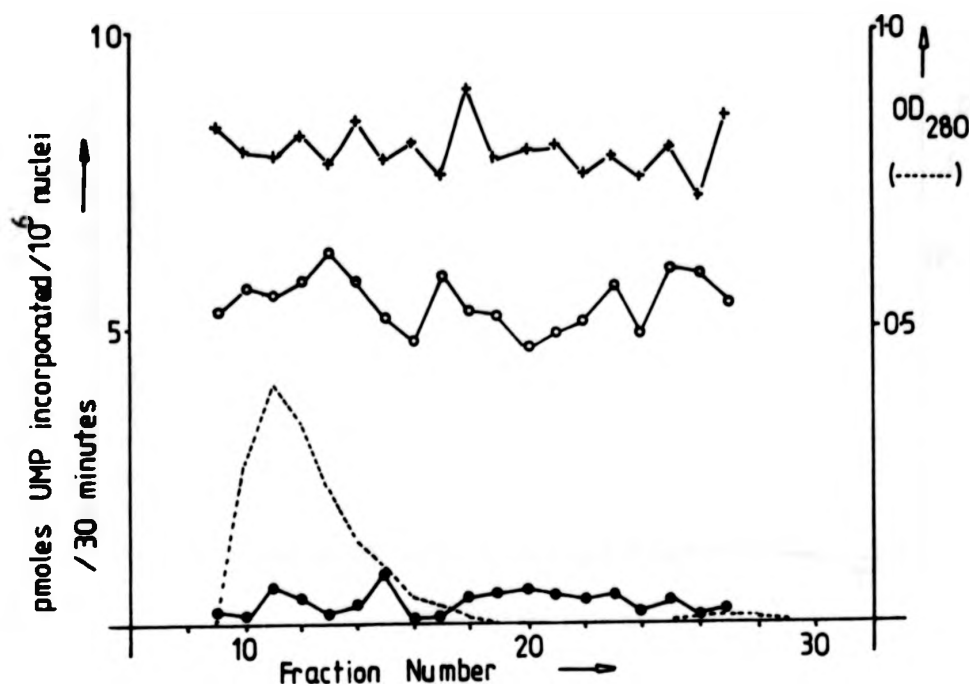


FIGURE 5.12

Fractionation by Sephadex G100 of an Early Cleavage Extract.

100 μ l of an early cleavage embryo extract was loaded onto a G100 column (2 cm diameter x 23 cm) and the resulting 30 drop fractions collected and stored at -20°C prior to being assayed. Three column assays were carried out, 5×10^5 nuclei were preincubated with 20 μ l column extract (o---o), 10 μ l column fraction and 10 μ l egg extract (+---+) or 10 μ l column fraction, 10 μ l egg extract and 100 $\mu\text{g/ml}$ α -amanitin (e---e). These were preincubated for 60 minutes, supplemented and incubated for 30 minutes.

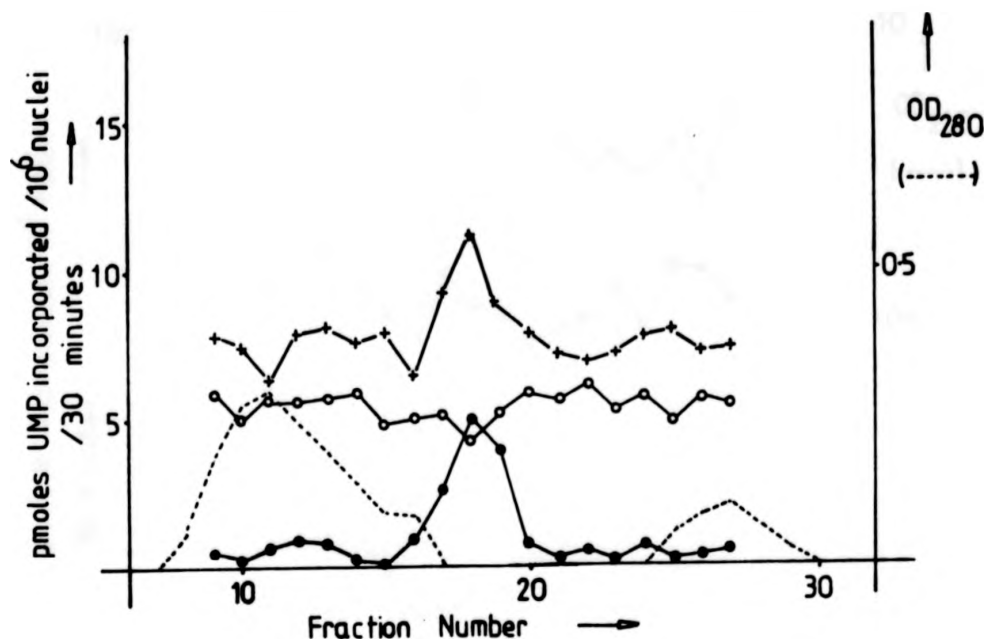


FIGURE 5.13

Fractionation by Sephadex G100 of a Gastrula Extract.

100 μ l of a gastrula extract was loaded onto a G100 column (2 cm diameter x 23 cm) and the resulting 30 drop fractions collected and stored at -20°C . Three column assays were carried out, 5×10^5 nuclei were preincubated with 20 μ l column fraction (o---o), 10 μ l column fraction plus 10 μ l egg extract (+---+) or 10 μ l column fraction, 10 μ l egg extract and 100 $\mu\text{g/ml}$ α -amanitin (●---●). These were preincubated for 60 minutes, supplemented and incubated for 30 minutes.

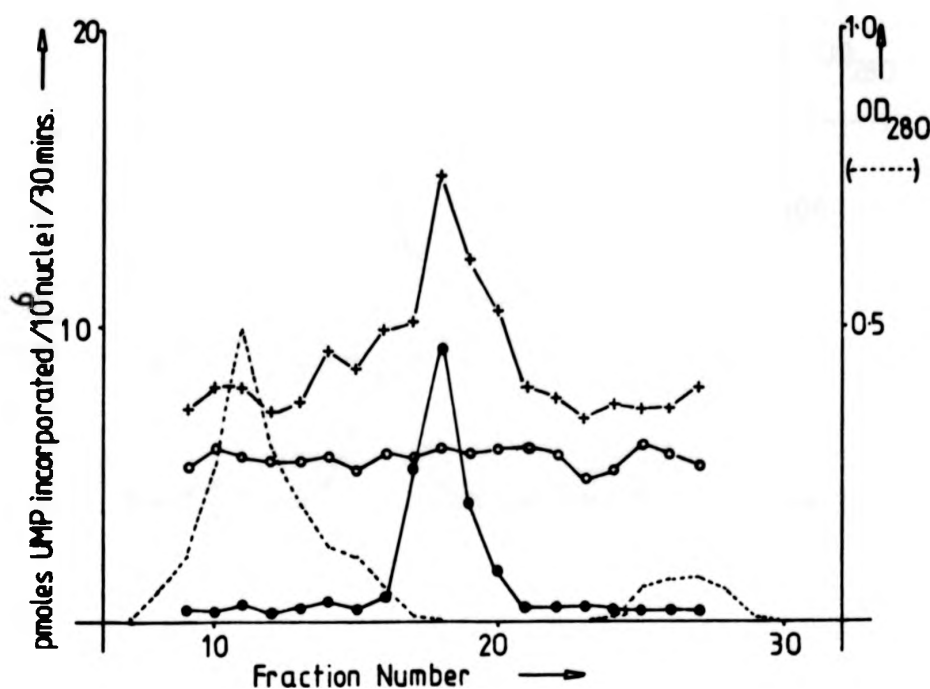


FIGURE 5.14

Fractionation by Sephadex G100 of a Neurula Extract.

100 μ l of a neurula extract was loaded onto a G100 column (2 cm diameter x 23 cm) and the resulting 30 drop fractions collected and stored at -20°C . Three column assays were carried out, 5×10^5 nuclei were preincubated with 20 μ l column fraction (o---o), 10 μ l column fraction and 10 μ l egg extract (+---+) or 10 μ l column fraction, 10 μ l egg extract and 100 $\mu\text{g/ml}$ α -amanitin (•---•). These were preincubated for 60 minutes, supplemented and incubated for 30 minutes.

peak of activity in any of the column fractions. This assay used egg extract as the RNA polymerase source, but the same result was obtained using the crude RNA polymerase preparation [FIG 5.11]. Even with increased sensitivity found by inhibiting RNA polymerases II and III with α -amanitin, no peak could be seen. Therefore there is no free activator protein as found in the oocyte extract. This kind of analysis shows only that the activity is absent, the protein could nevertheless be present in an inactive form.

Sephadex G100 Column Chromatography of Embryo Extracts.

Similar columns to those described above were run using a series of embryo extracts from early cleavage, gastrula and neurula stage embryos. They were assayed with or without α -amanitin using an egg extract as the RNA polymerase source. That from early cleavage embryos showed no peak of activity [FIG 5.12]. This lack of any stimulatory peak, similar to an egg column assay, is not surprising since the early cleavage extract has no effect on transcription in erythrocyte nuclei (Chapter IV). There would appear to be a slight peak of increased transcription in the fractions collected from a gastrula G100 column [FIG 5.13]. Although this activity appears to correspond to where the oocyte activating protein, Fr-18 would be and the transcription that results is resistant to α -amanitin, the amount of transcription is scarcely above background. In the neurula column fractions, however, a definite peak is seen [FIG 5.14]. Since the transcription seen is resistant to α -amanitin it is due to RNA polymerase I. The three sets of column fractions were also assayed using the crude RNA polymerase preparation instead of an egg extract. As Figures 5.15, 5.16 and 5.17 show, the results are

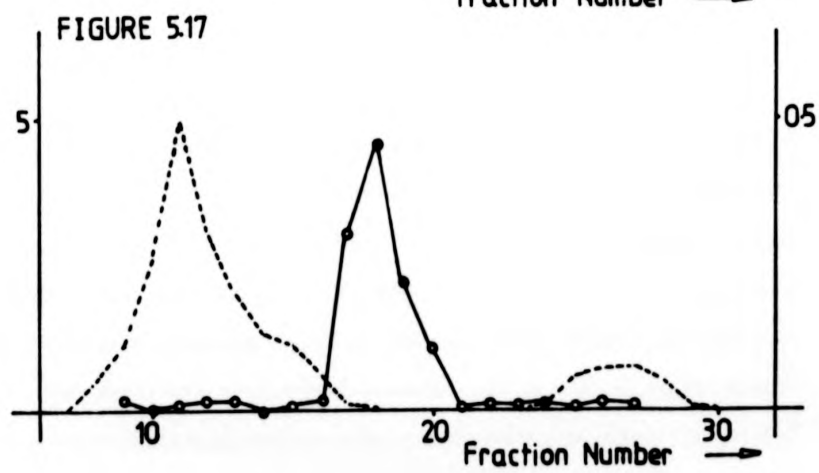
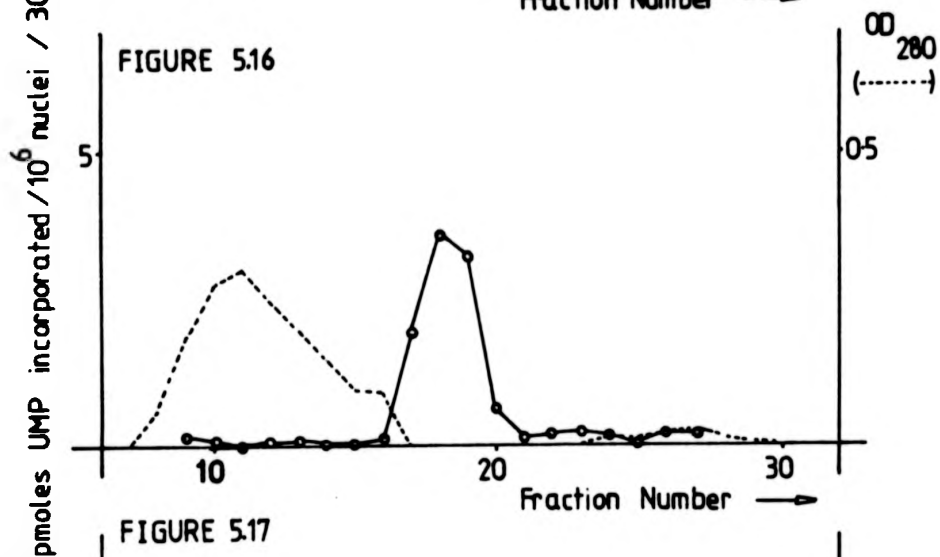
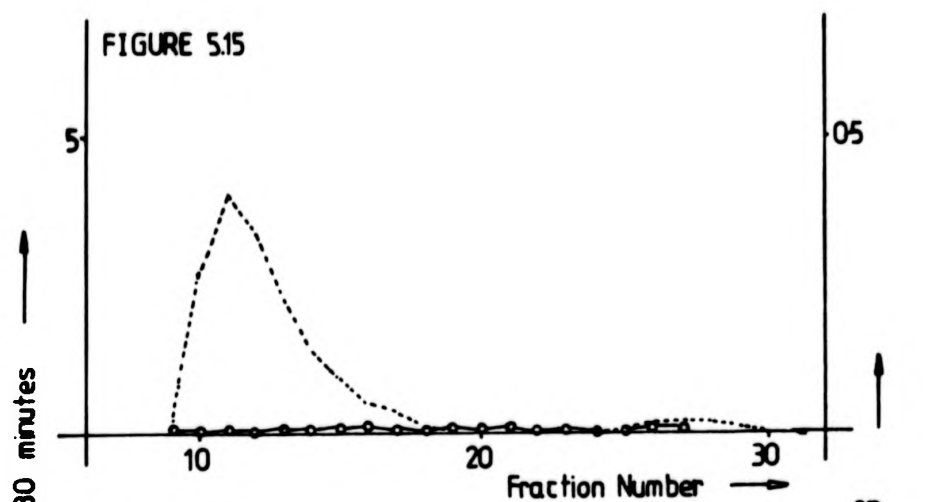


FIGURE 5.15

Early Cleavage G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.

15 μ l of the early cleavage G100 column fractions 9 to 27 were added to 5×10^5 nuclei and semipurified RNA polymerases with 100 μ g/ml α -amanitin in a final volume of 50 μ l such that only transcription by RNA polymerase I was possible. These were preincubated for 60 minutes, supplemented and incubated for a further 30 minutes.

FIGURE 5.16

Gastrula G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.

15 μ l of the gastrula G100 column fractions 9 to 27 were added to 5×10^5 nuclei and semipurified RNA polymerases with 100 μ g/ml α -amanitin in a final volume of 50 μ l such that only transcription by RNA polymerase I was possible. These were preincubated for 60 minutes, supplemented and incubated for a further 30 minutes.

FIGURE 5.17

Neurula G100 Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.

15 μ l of the neurula G100 column fractions 9 to 27 were added to 5×10^5 nuclei in a 50 μ l assay with semipurified RNA polymerases and 100 μ g/ml α -amanitin so that only RNA polymerase I transcription was possible. These were preincubated for 60 minutes, supplemented and incubated for a further 30 minutes.

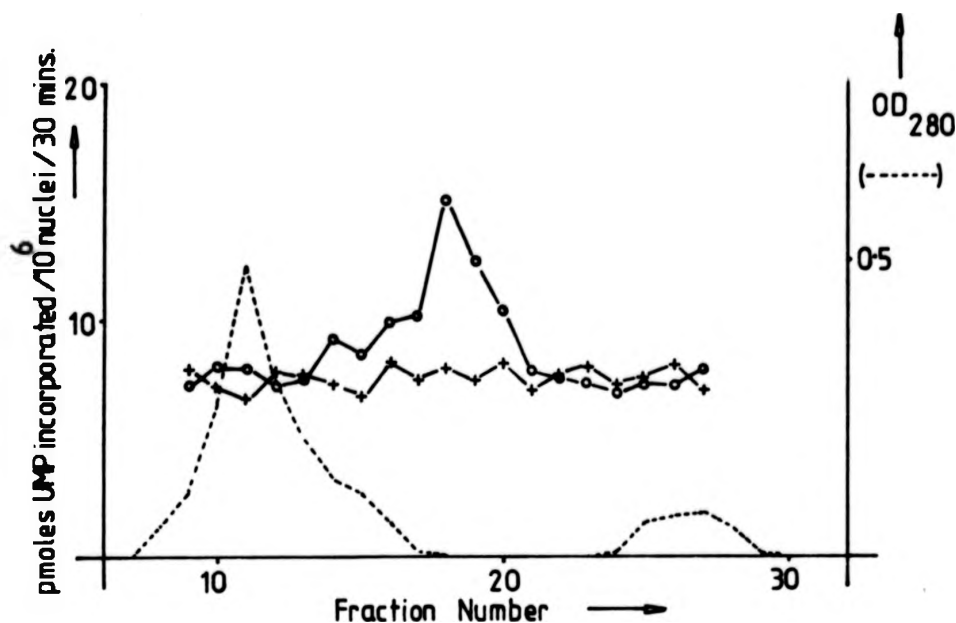


FIGURE 5.18

Heat Inactivation of "Fr-18" from Neurula G100 Column.

50 μ l of neurula G100 column fractions 9-27 were boiled for 10 minutes and cooled before 10 μ l of each was added to a standard 50 μ l column assay (+---+; 5×10^5 nuclei, 10 μ l egg extract). A parallel assay was carried out using unboiled column fractions (o---o). The assays were preincubated for 60 minutes, supplemented and incubated for a further 30 minutes. Duplicate 10 μ l samples were taken at the beginning and end of the incubation.

TABLE 5.3

Trypsin Inactivation of Active Component from a Neurula G100Column.

Preincubation Ingredients;	pmoles UMP incorp. /10 ⁶ nuclei/30 mins.
Nuclei + buffer	7.7
neurula G100 peak	15.1
boiled neurula G100 peak	7.6
trypsin x neurula G100 peak	7.2
trypsin-trypsin inhibitor x neurula G100 peak	14.9

100 μ l samples of neurula G100 fraction 18 were either boiled for 10 minutes, incubated with trypsin or incubated with trypsin and trypsin inhibitor. Trypsin inhibitor was added to the trypsin digestion after the 30 minute incubation. Erythrocyte nuclei were preincubated with 10 μ l aliquots of the above samples and 10 μ l egg extract. After the 60 minute preincubation the assays were supplemented with salts etc and incubated for a further 30 minutes.

Trypsin digestions were carried out as detailed in Table 5.2.

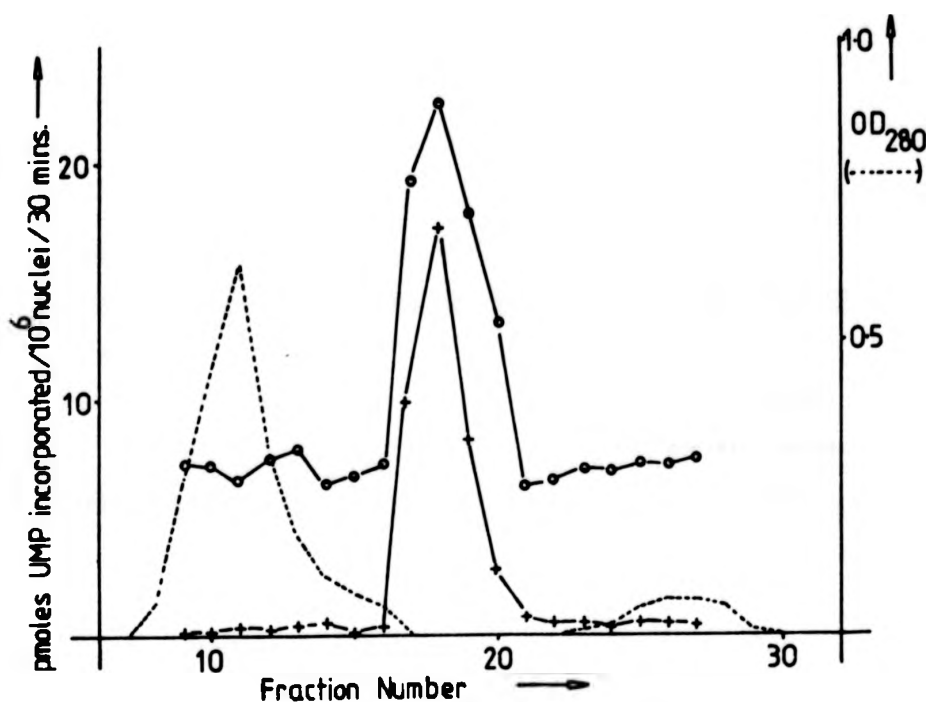


FIGURE 5.19

Fractionation by Sephadex G100 of a Combined Oocyte and Neurula Extract.

100 μ l of an oocyte and a neurula extract were combined and loaded onto a Sephadex G100 column (2 cm diameter x 23 cm), the 30 drop fractions were collected and stored at -20°C . These were assayed in two standard column assays (10 μ l column fraction, 10 μ l egg extract, 5×10^5 nuclei in a final volume of 50 μ l) with (+---+) or without (o---o) 100 $\mu\text{g/ml}$ α -amanitin. After a 60 minute preincubation the assays were supplemented and incubated for a further 30 minutes.

the same, indicating that the lack of any stimulatory proteins in early cleavage embryos is not an artefact from using an egg extract as the RNA polymerase source. As Figure 5.18 shows, the peak of transcriptional activity seen in fractions 17-19 of the neurula column are heat inactivated. Similarly, the peak's ability to permit RNA polymerase I transcription of erythrocyte chromatin is lost by trypsin digestion [TABLE 5.3]. Since this protein would appear to run in a corresponding place to Fr-18 isolated from an oocyte G100 column, the two proteins would appear to be very similar if not the same, in terms of molecular weight.

On combining an oocyte extract with a neurula extract, running the resulting mixture through a Sephadex G100 column and assaying in the normal manner, only one peak is found in fractions 17-19 [FIG 5.19]. It is no broader than that observed in oocyte or neurula unmixed columns, neither is there any evidence for a shoulder to this peak. Therefore, if these two proteins are not the same, then their molecular weights are very similar. The appearance of this protein, its lack in both egg and early cleavage extracts, its possible presence in the gastrula extract and its definite presence in neurula and oocyte extracts parallels the ability of the crude extracts to bring about RNA polymerase I activity on erythrocyte chromatin. Also it more-or-less mirrors the appearance of rRNA synthesis during embryonic development, although some workers (Shiokawa et al, 1981a, b) would set the beginning of rRNA synthesis earlier in mid to late blastula stage embryos. This apparent lag could be a reflection of the small amount of starting material in the case of the blastula embryo extract. Although de novo rRNA synthesis can be seen in these embryos, only a small percentage

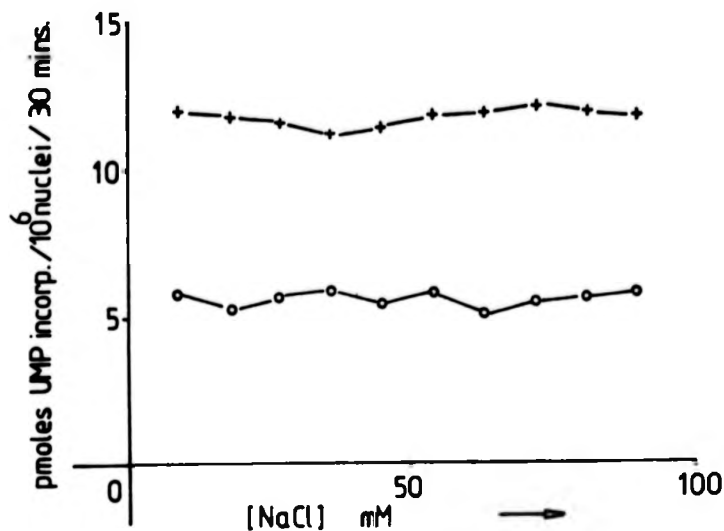


FIGURE 5.20

Effect of NaCl on Transcription by Erythrocyte Nuclei.

To a series of 50 μ l incubations (5×10^5 nuclei, 0.5xRB, 1 mM MnCl₂, 120 mM KCl, 0.4 mM ATP, CTP, GTP, 0.02 mM UTP and 2 μ Ci ³H-UTP) was added increasing amounts of NaCl from 0 to 90 mM. These were incubated for 30 minutes, duplicate 10 μ l samples being taken at 0 and 30 minutes.

(o---o) nuclei alone; (+---+) nuclei plus egg extract.

(Shiokawa et al, 1981a, b) of the embryonic cells are actually transcribing rRNA sequences. More cells start to synthesize rRNA as development proceeds (Woodland and Gurdon, 1968; Misumi et al, 1980; Shiokawa et al, 1981a, b).

Although a protein that affects RNA polymerase I activity in erythrocyte nuclei can be identified on an oocyte or neurula G100 column, no fraction was found that affected RNA polymerases II and III. So whilst incubation with an oocyte or a neurula extract brings about an increase in the activity of all the RNA polymerases, no protein was isolated that affected RNA polymerases II or III from these columns. The observed stimulation of these RNA polymerases by oocyte and neurula extracts could be produced by agents also present in the egg and RNA polymerase preparation.

DEAE Cellulose Chromatography of Oocyte Extracts.

A Sephadex G100 column will only separate proteins on the basis of their size. In order to determine whether there is one or more active protein types in Fr-18 and whether this is the same as that found in the neurula column fractions at the same place, another method of column chromatography was used, that of DEAE cellulose, which depends on charge.

An oocyte extract was loaded on a DEAE cellulose column and all the non-binding proteins washed off before a NaCl gradient was applied. Figure 5.20 demonstrates that increasing the Na concentration has little or no effect on transcription by erythrocyte nuclei with or without an egg extract present. This is somewhat surprising since the monovalent cation included in

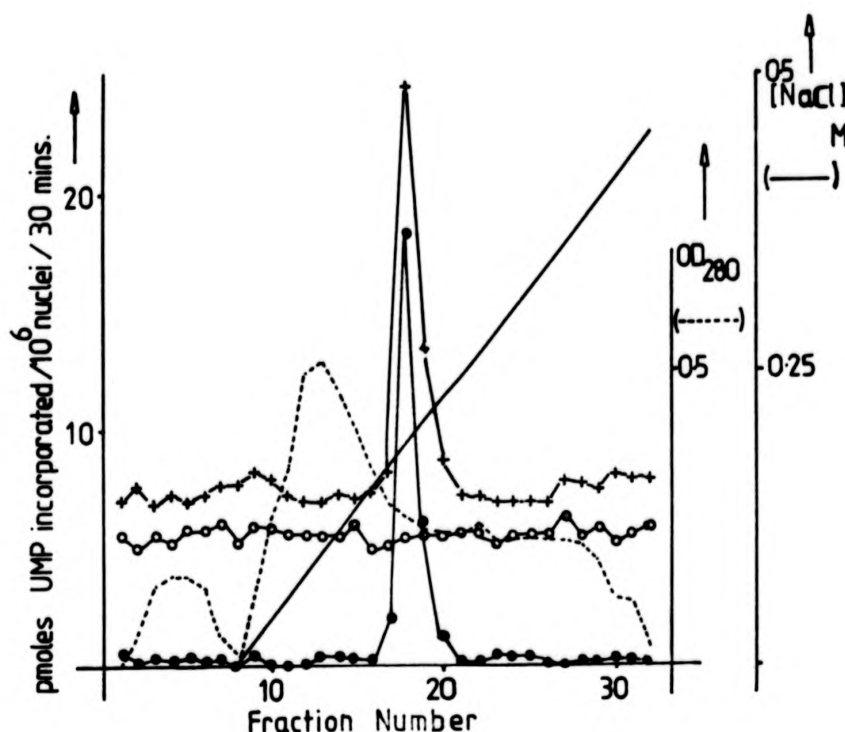


FIGURE 5.21

Fractionation by DEAE cellulose of an Oocyte Extract.

A 300 μ l sample of an oocyte extract was loaded onto a DEAE cellulose column (2 cm diameter x 2.5 cm) which was washed through with column buffer until the OD of the eluate was zero. A 0-0.45 NaCl gradient was passed through the column, 30 drop fractions collected and stored at -20°C . These were assayed in a standard 50 μ l column assay with either erythrocyte nuclei alone (o---o), erythrocyte nuclei plus egg extract (+---+) or erythrocyte nuclei, egg extract and 100 $\mu\text{g/ml}$ α -amanitin (e---e). The assays were preincubated for 60 minutes, supplemented and incubated for 30 minutes.

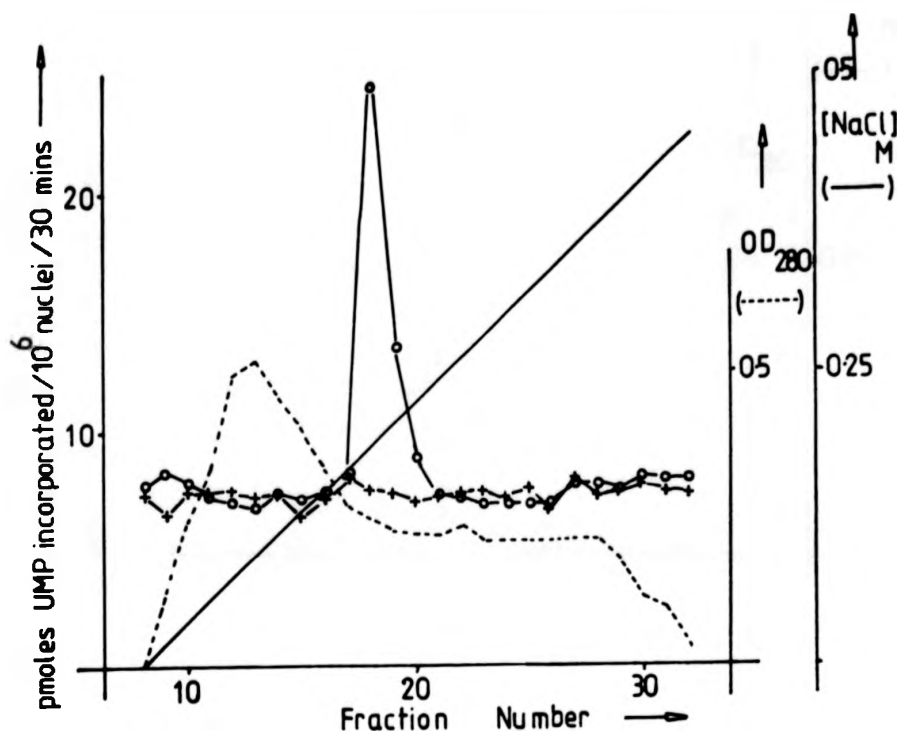


FIGURE 5.22

Heat Inactivation of the Active Component Separated on a DEAE
cellulose Column.

50 μl samples of oocyte DEAE cellulose column fractions 8-32 were boiled for 10 minutes and cooled before 10 μl of each was added to a standard 50 μl column assay (5×10^5 nuclei, 10 μl egg extract; $+--+$). A parallel assay was carried out using unboiled column fractions (\circ --- \circ). Both assays were preincubated for 60 minutes, supplemented and incubated for a further 30 minutes.

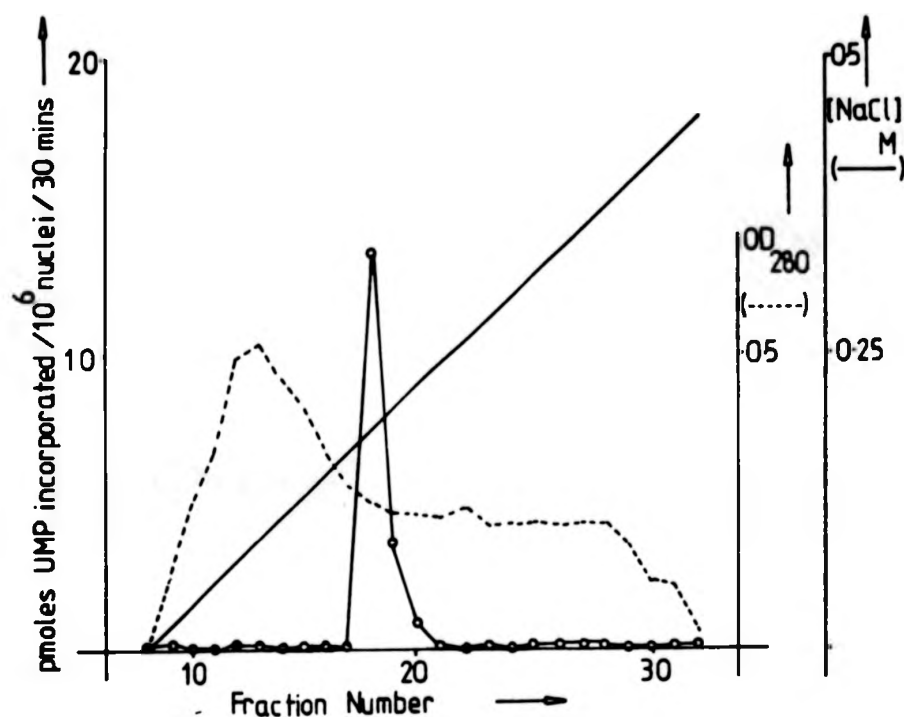


FIGURE 5.23

Oocyte DEAE Cellulose Column Assay with Erythrocyte Nuclei and Semipurified RNA Polymerases.

15 μ l of fractions 8-32 from an oocyte DEAE cellulose column were added to 5×10^5 nuclei, semipurified RNA polymerases and α -amanitin at 100 μ g/ml in a final volume of 50 μ l. Only transcription by RNA polymerase I was possible. These were preincubated for 60 minutes, supplemented and incubated for a further 30 minutes.

TABLE 5.4

Inactivation of Active Component from an Oocyte DEAE Column.

Preincubation Conditions;	pmoles UMP incorp. /10 ⁶ nuclei/30 mins.
Nuclei + buffer	7.8
oocyte DEAE peak	19.5
boiled oocyte DEAE peak	7.6
trypsin x oocyte DEAE peak	6.9
trypsin-trypsin inhibitor x oocyte DEAE peak	20.1

100 μ l samples of pooled fractions 18 and 19 from an oocyte DEAE column [FIG 5.21] were either boiled for 10 minutes, incubated with trypsin or trypsin plus trypsin inhibitor. Trypsin inhibitor was added to the trypsin digestion after the 30 minute incubation. Erythrocyte nuclei were preincubated with 10 μ l aliquots of the above samples, or untreated DEAE pooled fractions 18-19, and 10 μ l egg extract. After the 60 minute preincubation, the assays were supplemented with salts etc and incubated for a further 30 minutes.

Trypsin digestions were carried out as detailed in Table 5.2.

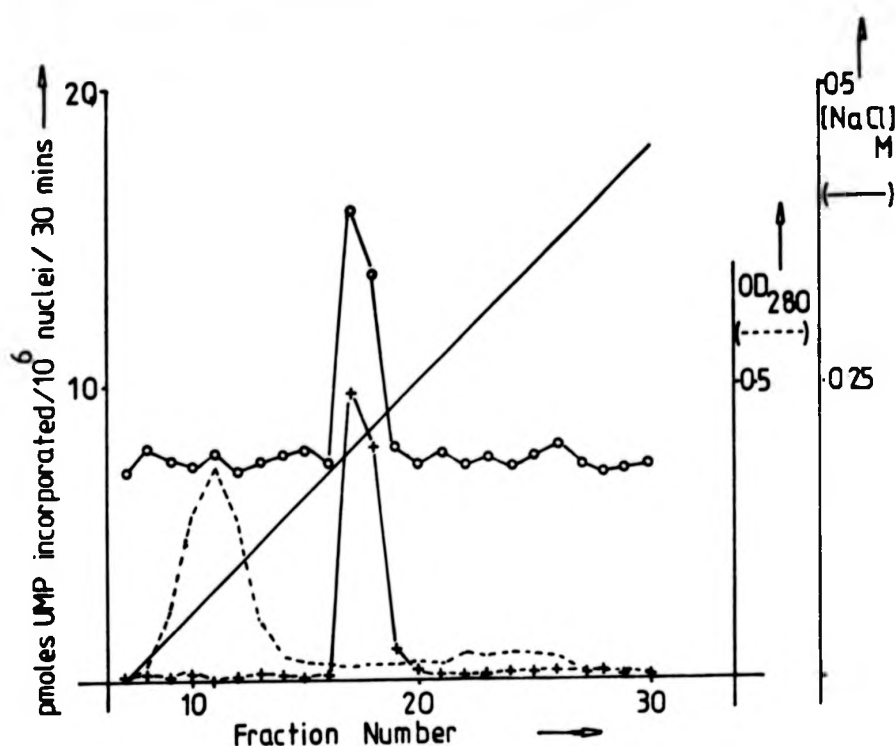


FIGURE 5.24

Fractionation by DEAE cellulose of Fr-18.

A 3 ml sample of pooled fractions 17-19 from an oocyte G100 column were loaded onto a DEAE cellulose column (2 cm diameter x 2.5 cm). The column was washed with column buffer before the NaCl gradient was applied and the resulting 30 drop fractions collected and stored at -20°C. 10 µl samples of these fractions were added to 50 µl column assays (5x10⁵ nuclei, 10 µl egg extract) either without (o---o) or with (+---+) α-amanitin at 100 µg/ml. the assays were preincubated for 60 minutes before being supplemented and incubated for a further 30 minutes.

the basic transcription recipe, potassium, has a sharp optimal concentration (FIG 3.2A), and sodium ions are not normally included. The column fractions from the DEAE column were assayed using an egg extract as the polymerase source (FIG 5.21). Only a single peak of increased transcription was seen, it was eluted from the column by 190 mM NaCl just after the majority of the bound oocyte proteins have been eluted. By the addition of α -amanitin to the column assay it can be shown that the increased transcription is due to RNA polymerase I [FIG 5.21]. There is no sign of any increase in the activity of the other two RNA polymerases, and incubating the column fractions with erythrocyte nuclei alone has no effect on transcription [FIG 5.21]. To check that the increased transcriptional activity is not an artefact of the salt concentration, the column fractions were dialysed against Na-free column buffer and then assayed in the normal way. As can be seen [FIG 5.22], the peak of increased, α -amanitin resistant, transcriptional activity is still present in fractions 18 and 19. This peak is still present if the egg extract is replaced by the crude RNA polymerase preparation [FIG 5.23]. Table 5.4 shows that the active component of fractions 18 and 19 of the DEAE salt gradient is a protein, since it is heat degradable and trypsin sensitive.

To determine whether the oocyte protein(s) isolated from the G100 column is the same as that isolated from the DEAE column, a sample of pooled fractions 17-19 from the G100 column was loaded onto a DEAE column and salt eluted. Only one peak, eluted by 190 mM NaCl, was shown by the transcription assay [FIG 5.24]. It is eluted from the column by the same concentration of NaCl as the protein from the total oocyte extract. The amount of stimulation observed on incubating these column fractions (fractions 17-18)

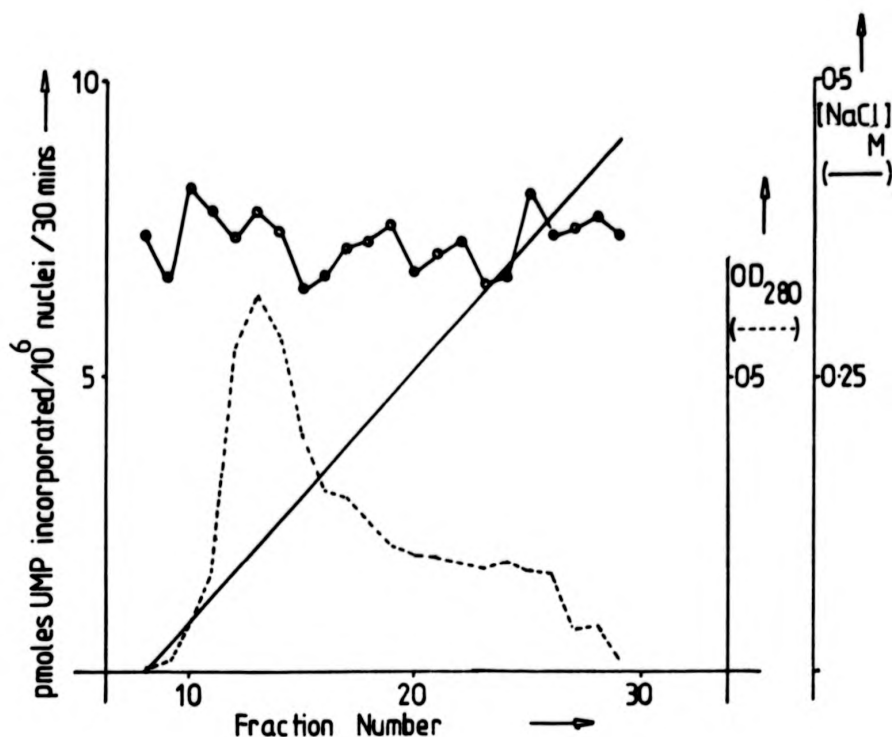


FIGURE 5.25

Fractionation by DEAE cellulose of an Egg Extract.

A 300 μ l sample of an egg extract was loaded onto a DEAE cellulose column (2 cm diameter x 2.5 cm). The column was washed with column buffer before the NaCl gradient was applied and the resulting 30 drop fractions collected and stored at -20°C . 10 μ l samples of these fractions were added to 50 μ l column assays (5×10^5 nuclei, 10 μ l egg extract; o---o). The assays were preincubated for 60 minutes, supplemented and incubated for 30 minutes.

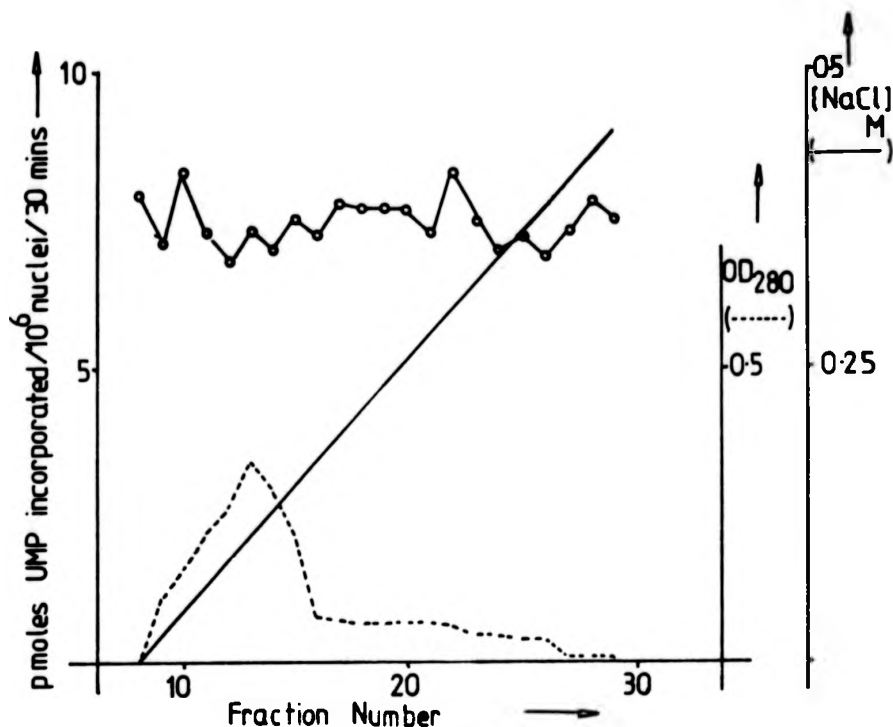


FIGURE 5.26

Fractionation by DEAE cellulose of Pooled Fractions 17-19 from an Egg G100 Column.

3 ml of the pooled egg G100 column fractions were loaded onto a DEAE cellulose column (2 cm diameter x 2.5 cm). The column was washed with column buffer before the NaCl gradient was applied and the resulting 30 drop fractions collected and stored at -20°C. 10 µl samples of the fractions were added to a standard column assay (10 µl column fraction, 10 µl egg extract, 5x10⁵ nuclei in a final volume of 50 µl). These were preincubated for 60 minutes, supplemented and incubated for 30 minutes.

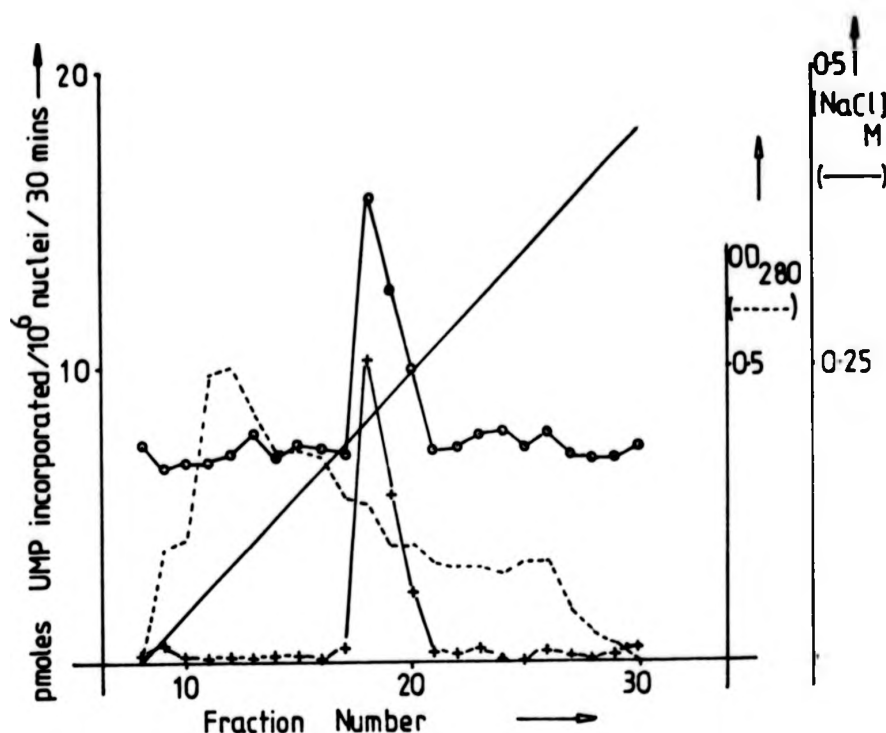
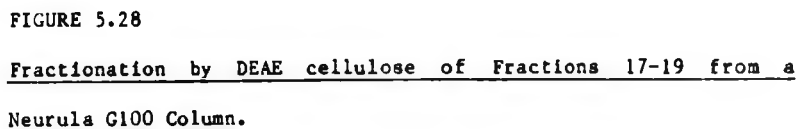


FIGURE 5.27

Fractionation by DEAE cellulose of a Neurula Extract.

A 150 μ l sample of a neurula extract was loaded onto a DEAE cellulose column (2 cm diameter x 2.5 cm). This was washed with column buffer before the NaCl gradient was applied, the resulting 30 drop fractions were collected and stored at -20°C . 10 μ l samples of these fractions were added to a standard column assay (5×10^5 nuclei, 10 μ l column fraction, 10 μ l egg extract) with (+---+) or without (o---o) α -amanitin at 100 $\mu\text{g}/\text{ml}$. These were preincubated for 60 minutes, supplemented and incubated for 30 minutes.



A 3 ml sample of the pooled fractions was loaded onto a DEAE cellulose column (2 cm diameter x 2.5 cm) which was washed with column buffer before the NaCl gradient was applied. 30 drop fractions were collected and stored at -20°C. 10 µl of these fractions were added to a standard column assay (5x10⁵ nuclei, 10 µl egg extract, 10 µl column fraction) with (+---+) or without (o---o) 100 µg/ml α-amanitin. These were preincubated for 60 minutes, supplemented and incubated for 30 minutes.

with erythrocyte nuclei is less than that seen with Fr-18, or fractions 18-19 from a total oocyte DEAE column. However, this can be explained by the two-fold dilution that has taken place on running the column. By these purification criteria it would appear that only a single protein is responsible for inducing RNA polymerase I transcription on erythrocyte chromatin.

DEAE Cellulose Salt Elution of Egg and Neurula Extracts.

The peak of transcriptional activity seen in oocyte extracts fractionated on DEAE cellulose [FIG 5.21] was absent if the same experiment was carried out using an egg extract [FIG 5.25]. This agrees well with the data from the G100 column. Neither is a peak of transcriptional activity seen if fractions 17-19 from an egg G100 column were fractionated on a DEAE column [FIG 5.26]. Thus if an inhibitor is present in the egg extract and coupled in some way with the activator molecule, it must either remain bound to it during Sephadex and DEAE chromatography or must co-elute with the stimulatory agent in both procedures.

As expected, the neurula extract which yields a stimulatory peak on passage through Sephadex G100 also yields a stimulatory peak on salt elution from DEAE cellulose [FIG 5.27]. Like Fr-18 from oocyte extracts, this is eluted by 190 mM NaCl. The neurula G100 fractions 17-19 when salt eluted from DEAE cellulose also show a single peak of transcriptional activity at 190 mM NaCl [FIG 5.28]; the smaller stimulation was due to dilution effects and the small amount of starting material. Both transcription peaks, from crude neurula extract or the semipurified G100 fractions, are a result of RNA polymerase I activity as judged by their resistance to 100 µg/ml α -amanitin [FIGS 5.26, 5.27].

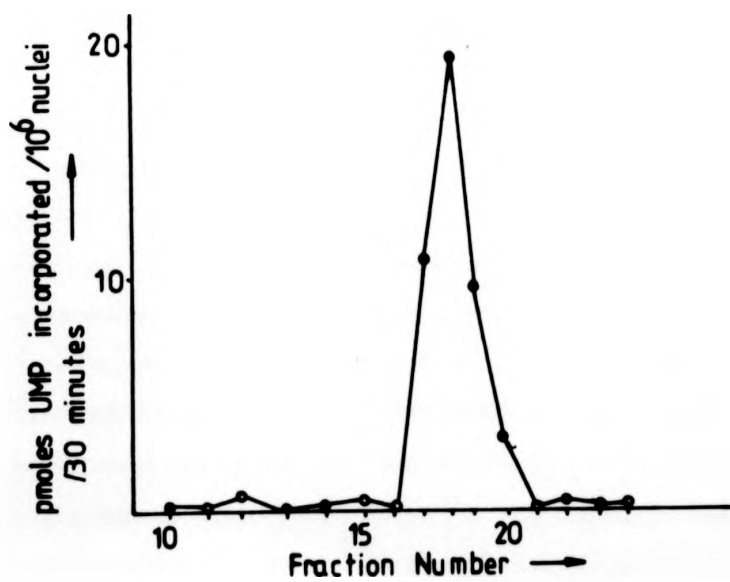
Fraction Number;

10

15

20

25



Fraction Number;

10 15 20 25

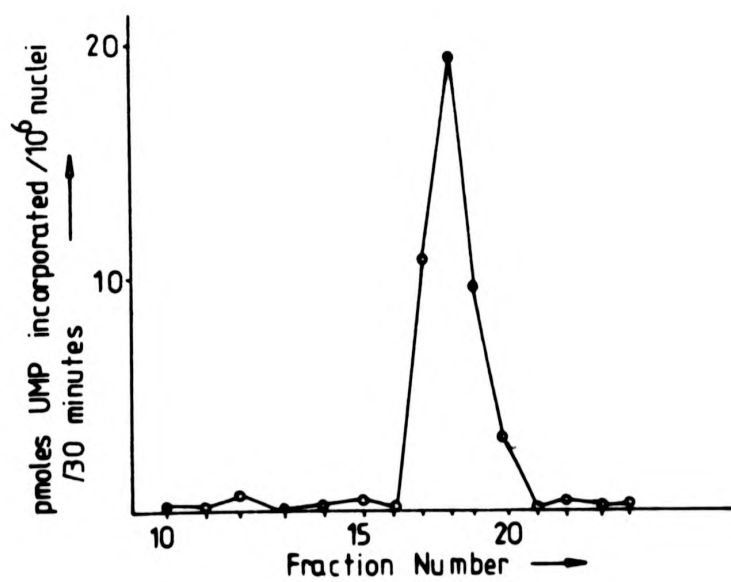
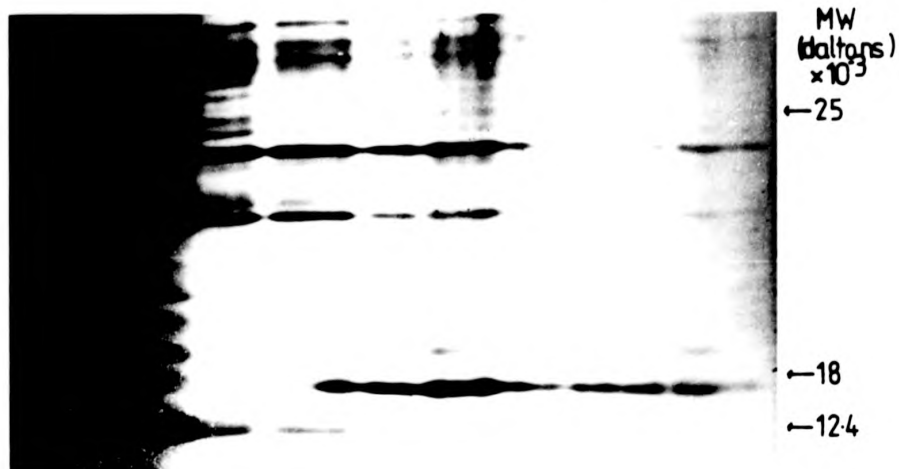


FIGURE 5.29

Acrylamide Gel Analysis of Oocyte G100 Column Fractions.

200 μ l of each column fraction was dried down before being taken up in 10 μ l sample buffer and loaded onto a 18% low bis acrylamide gel. This was run overnight until the marker dye had migrated to the bottom of the gel. The gel was fixed, silver stained (Switzer et al, 1979) and photographed. The data for the column assay is that from Figure 5.2.

Oocyte

Egg

Neurula

MW $\times 10^{-3}$

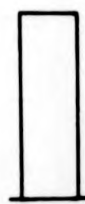
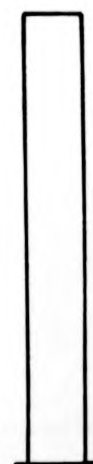
25

17.8

124

pmoles UMP incorporated / 10^6 nuclei / 30 mins.

20
10



Oocyte

Egg

Neurula

MW $\times 10^{-3}$

← 25

← 17.8

← 124

pmoles UMP incorporated / 10^6 nuclei
/ 30 mins.

20

10

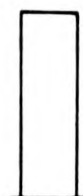
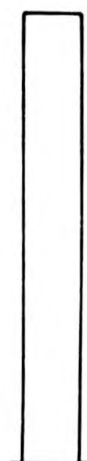


FIGURE 5.30

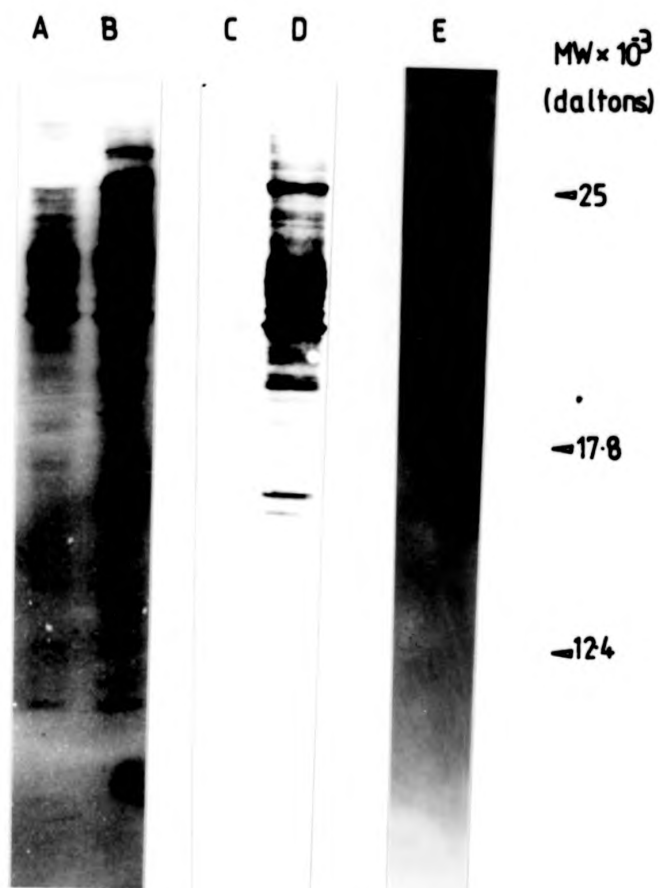
Acrylamide Gel Analysis of Proteins in Fractions 17-19 of
Oocyte, Egg and Neurula G100 Columns.

200 μ l of pooled fraction 17-19 from an oocyte, egg and neurula G100 column were freeze dried before being taken up in 10 μ l sample buffer and loaded onto a 18% low bis acrylamide gel. This was run and stained as in Figure 5.29. The data for the RNA polymerase I activity in erythrocyte nuclei preincubated with an egg extract and these column fractions are those from Figures 5.2, 5.10 and 5.18.

Visualization on Acrylamide Gels of the Oocyte Stimulatory Protein, Fr-18.

It is possible that the protein responsible for the RNA polymerase I transcription is a minor one within the oocyte extract. For instance, if there was one of these protein molecules (of molecular weight 20,000 daltons) for each of the 1.5-2.5 million ribosomal genes (Perkowska et al, 1968) present in the oocyte, this would only amount to 0.07 pg of Fr-18/oocyte. This is, however, a minimum estimate since it is likely that there is more than one Fr-18 molecule per ribosomal RNA gene in the cell. For maximum sensitivity, the silver staining technique of Switzer et al, (1979) was used on oocyte G100 column fractions that had been analysed on acrylamide gels. Oocyte extracts contain a multitude of different sized proteins, several of which are present in fraction 18 [FIG 5.29]. It is likely that Fr-18 is present in oocyte and neurula extracts, but absent from egg extracts. Only one protein that is abundant in fraction 18 shows this distribution [FIG 5.30]. From the mobility of marker proteins, this protein has a molecular weight of 21,000-22,500 daltons.

The evidence for this protein seen on the stained acrylamide gels being Fr-18 is circumstantial and not rigorous. Silver staining of all column fractions from an oocyte DEAE cellulose column was attempted but no proteins at all were visible from fraction 15 onwards, and the high concentration of salt coupled with the low concentration of protein made analysis difficult.



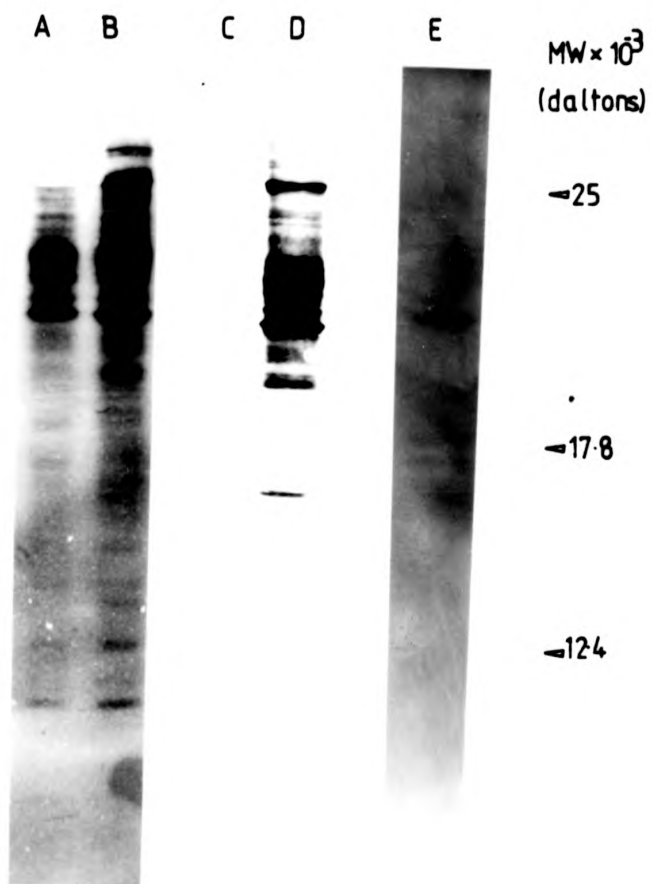


FIGURE 5.31

Migration of Oocyte Proteins into Erythrocyte Nuclei.

Erythrocyte nuclei in a standard 50 μ l assay (5×10^5 nuclei) were preincubated with either 20 μ l of a 35 S-oocyte extract (Track D), or 20 μ l of Fr-18 (Track E) from a 35 S-oocyte G100 column. After a 60 minute preincubation the nuclei were spun out of the buffer washed twice with 100 μ l of RB, and taken up in sample buffer. These were loaded onto a 18% low bis acrylamide gel which was run in the normal manner, fixed and stained with Coomassie blue and fluorographed before exposure to an X-ray film. 20 μ l 35 S-oocyte extract was also preincubated with no nuclei (Track C), but spun, washed and loaded onto the gel in the same way. Also loaded were samples of the 35 S-oocyte extract (Track A) and separated germinal vesicles (Track B).

Migration of Fr-18 into Erythrocyte Nuclei.

For Fr-18 to bring about transcription by RNA polymerase I either it must pass into the erythrocyte nuclei or it must exert its effect on the RNA polymerase I molecules themselves before they enter the erythrocyte nuclei. If the latter is the case, this modification is lost relatively easily, since partially purified RNA polymerase I molecules from the ovary do not transcribe erythrocyte chromatin. In the former case, it is possible that the protein acts either on the RNA polymerase I molecules, eg by loosely binding to them, or on the ribosomal genes, perhaps by altering their configuration to allow transcription to take place.

An ^{35}S -labelled oocyte extract was made by incubating vitellogenic oocytes in ^{35}S -methionine and processing them in the usual way. Nuclei were preincubated with the ^{35}S -oocyte extract in a standard preincubation buffer for 60 minutes, the nuclei were then isolated and washed to remove any proteins that might have stuck unspecifically to the nuclei. If this procedure was carried out with only the ^{35}S -oocyte extract present and no nuclei, only 0.002% of the available counts were left. The proteins extracted from the nuclei were run out on an acrylamide gel, fluorographed and exposed to an X-ray film. About 28% of the available counts in the oocyte extract become associated with the erythrocyte nuclei and these counts represent a number of proteins of differing molecular weights [FIG 5.31]. The ^{35}S -oocyte extract was fractionated on G100 Sephadex, and nuclei preincubated with each fraction in the same way as with the total extract. A certain amount of the radioactivity from the main column peak becomes associated with the erythrocyte nuclei [FIG 5.32], but this

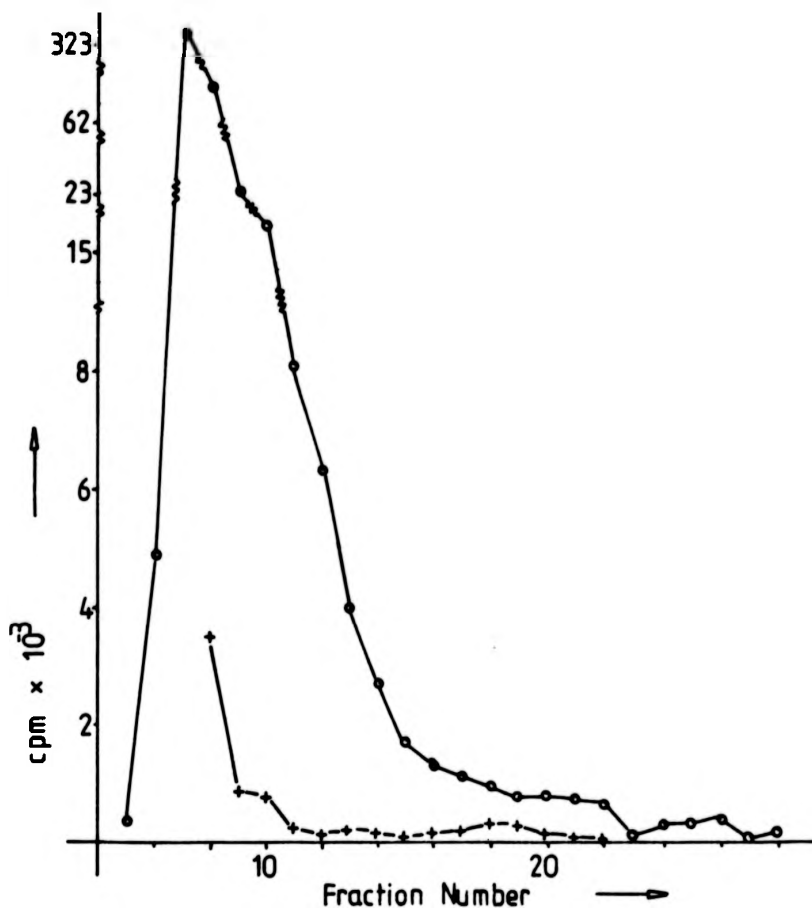


FIGURE 5.32

Migration of Oocyte Proteins into Erythrocyte Nuclei.

A 200 μ l sample of the ³⁵S-oocyte extract was loaded onto a G100 column (2 cm diameter x 23 cm) and the 30 drop fractions collected and stored at -20°C. 20 μ l samples of these fractions were preincubated with 5×10^5 nuclei in a standard 50 μ l column assay. After the 60 minute preincubation the nuclei were spun down, washed twice with RB and taken up in 20 μ l RB. Duplicate 10 μ l samples from each were counted (+---+), as were 10 μ l samples of each column fraction (o---o).

represents only 5.6% of the counts available. When nuclei were incubated with the fraction corresponding to fraction 18 of the original oocyte G100 columns, 27% of the counts available associate with the nuclei [FIG 5.32] as compared with about 6% in the surrounding fractions. It would appear therefore, that a protein (or proteins) present in fraction 18, becomes associated with the erythrocyte nuclei during the preincubation stage of the transcription assay and remains there.

The proteins extracted from nuclei preincubated in this way were analysed on acrylamide gels as before. Nuclei preincubated with ³⁵S-fraction 18 proteins only retain a single protein [FIG 5.31], this band is seen when nuclei are preincubated with total S-oocyte extract. From marker proteins run on the gels, this protein has a molecular weight of 21,000-22,500 daltons. Its migration through the acrylamide gel corresponds to that of the protein, present in oocytes and neurula embryos but not eggs, suggested by silver staining [FIG 5.30]. This protein was labelled by incubation of large oocytes with ³⁵S-methionine and therefore must be synthesized during late oogenesis inspite of the fact that the amplified rDNA is activated at mid-oogenesis. So it might be that Fr-18 has a relatively short half life as compared to the time taken to transcribe the oogenetic rRNA and the protein is required throughout this stage.

Effect of Fr-18 on Transcription by Erythrocyte Nuclei.

Incubation of erythrocyte nuclei with an oocyte extract brings about newly initiated DNA-dependent RNA polymerase I transcription. It will be shown in Chapter VI that the product of this transcription is ribosomal RNA. The effect of Fr-18 is

TABLE 5.5

Effect of Rifamycin AF/013 and Actinomycin D on Transcription by Erythrocyte Nuclei Preincubated with Egg Extract and Fr-18.

Preincubation Conditions	pmoles UMP incorp. /10 ⁶ nuclei/30 mins.
Nuclei + buffer	5.7
egg extract	12.6
oocyte extract	33.6
Fr-18	5.5
egg extract + Fr-18	23.7
egg extract, Fr-18 + Actinomycin D	0.02
egg extract, Fr-18 + Rifamycin AF/013	0.06

Erythrocyte nuclei (5×10^5 nuclei/50 μ l assay) were preincubated with 20 μ l buffer, egg extract, oocyte extract or Fr-18. Similar preincubations were carried out with 10 μ l egg extract, 10 μ l Fr-18 and either 100 μ g/ml Actinomycin D, 50 μ g/ml Rifamycin AF/013 or neither. After the 60 minute preincubations the assays were supplemented and incubated for a further 30 minutes.

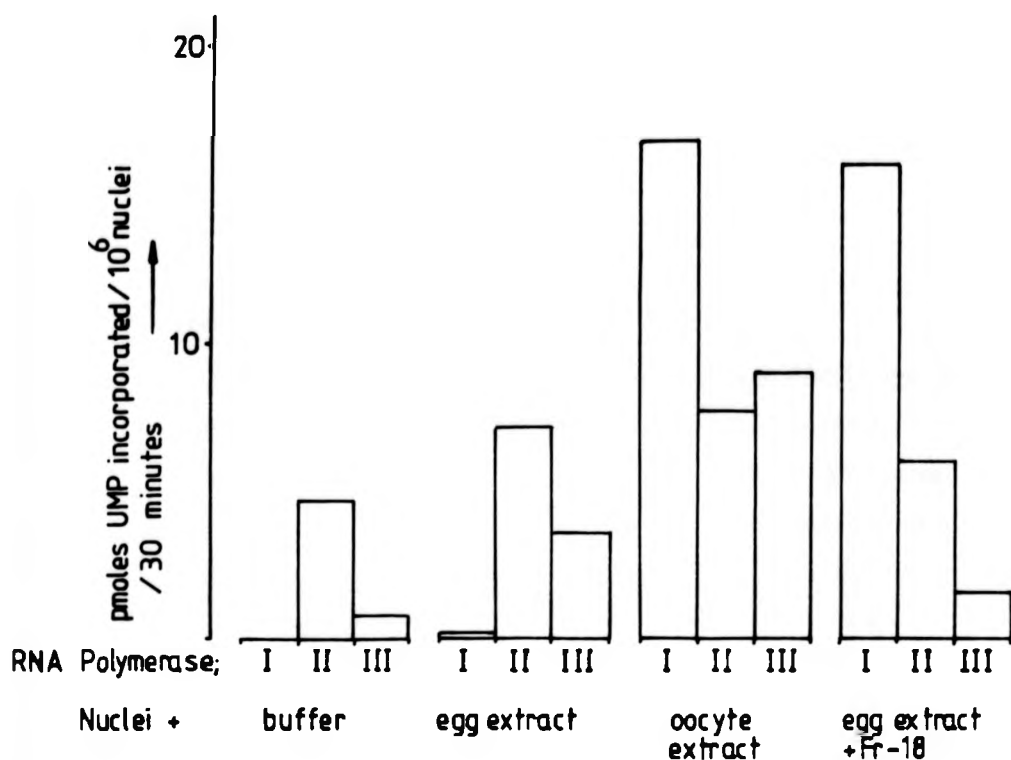


FIGURE 5.33

Relative Levels of the Three RNA Polymerases in Nuclei
Preincubated with Egg Extract and Fr-18.

Duplicate 50 μ l assays (5×10^5 nuclei) were preincubated with 20 μ l buffer, egg extract or oocyte extract, or 10 μ l egg extract and 10 μ l Fr-18. with 0, 1 or 100 μ g/ml α -amanitin. After 60 minutes the assays were supplemented and incubated for a further 30 minutes.

TABLE 5.6

Time of Addition of Fr-18.

Ingredients During;		pmoles UMP incorp.
Preincubation	Incubation	/10 ⁶ nuclei/30 mins.
egg extract + Fr-18	egg extract + Fr-18	23.0
buffer	egg extract + Fr-18	19.6
Fr-18	egg extract + Fr-18	22.7
egg extract	egg extract + Fr-18	18.8
egg extract	egg extract	10.3
Fr-18	Fr-18	5.3
Fr-18	egg extract	22.9

5
 5x10⁵ nuclei were preincubated with buffer, Fr-18 and egg extract as in the table above in a final volume of 50 μ l. After 60 minutes the nuclei were spun down, washed in RB and taken up in an incubation mix as outlined above. A 30 minute incubation followed.

identical to that seen when the total oocyte extract is used, provided, of course, that RNA polymerase I is also present. On being assayed with an egg extract and Fr-18, erythrocyte nuclei show a 6-fold stimulation in total transcription, 67% of which, as judged by α -amanitin inhibition, is as a result of RNA polymerase I activity [FIG 5.33]. Incubation with just an egg extract or Fr-18 yields no such increase in transcription [TABLE 5.5]. The RNA polymerase I transcription is sensitive to both Rifamycin SV and actinomycin D indicating that the RNA polymerase I is initiating new chains on a DNA template [TABLE 5.5]. However, unlike the oocyte extract, the addition of Fr-18 plus excess RNA polymerases has no effect on the activity of RNA polymerases II and III [FIG 5.33].

Although in a standard assay both RNA polymerase and Fr-18 are present during an incubation, it is not necessary for the RNA polymerase I molecules to be present during the preincubation step [TABLE 5.6], only Fr-18. If the RNA polymerases are added at the end of the preincubation the same stimulation is seen as when they were added at the beginning. However, when Fr-18 is added at the end of the preincubation, the amount of RNA polymerase I transcription is as if there were no preincubation. The degree of RNA polymerase I transcription is therefore dependent on the time that the active component of Fr-18 is present with the nuclei. It therefore seems unlikely, especially as the copurifying band from the silver stained column fractions appears to become associated with the nuclei, that the active component is modifying the RNA polymerase I molecules before they enter the nuclei. It is more likely that it modifies the ribosomal genes in some way.

CHAPTER VI; FIDELITY OF TRANSCRIPTION BY RNA POLYMERASE I ON
ERYTHROCYTE NUCLEAR CHROMATIN

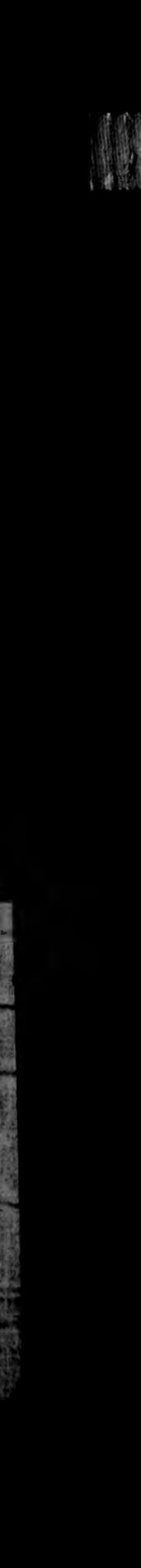
In vivo RNA polymerase I transcribes the ribosomal genes to produce a 40S precursor molecule, later cleaved to produce the 18S, 28S and 5.8S rRNA species. It does not transcribe any other sequence and is located exclusively in the nucleoli of active cells (Roeder and Rutter, 1970). The remaining RNA transcription is carried out by RNA polymerase II to produce hnRNA and mRNA, and by RNA polymerase III which transcribes the 4S (tRNA) and 5S gene sequences. So although the 40S and 5S RNA species are all ribosome components, the two RNA species are transcribed by different RNA polymerases and are under different control mechanisms. The protein described by Engelke et al (1980) seems to be specific to 5S gene transcription and to have no effect on other gene sequences.

However specific RNA polymerase I may be for the ribosomal genes of intact cells, its specificity may not be maintained in an in vitro transcription system. Although the erythrocyte nuclear transcription assay could be considered to be nearer the in vivo situation than if a recombinant DNA clone was being used as template, it cannot be regarded as a true reflection of the in vivo system for a number of reasons. Erythrocyte nuclei do not normally meet oocyte cytoplasm in a frog, and more importantly, the mature erythrocyte does not have transcribing ribosomal genes. Although RNA polymerase I has been shown (Grummt, 1981; Grummt et al, 1982) to transcribe recombinant ribosomal genes accurately, it is possible that in my system it is transcribing unspecifically. Specificity can be shown by demonstrating that not only is the RNA produced read from the

correct strand, but also that initiation and termination occurs at the correct points and that non-transcribed spacer regions between the genes are not transcribed.

The ribosomal genes in Xenopus laevis are tandemly repeated 470-fold per haploid genome (Brown and Weber, 1968; MacGregor, 1968; Gall, 1969) and have a repeat length of about 11.43 kb. The repeat itself consists of about 3.59 kb of non-transcribed spacer and 7.84 kb of transcribed sequence [FIG 6.1] (Boseley et al, 1978; 1979) The actual 40S precursor RNA molecule contains the 18S and 28S rRNA molecules plus a small 5.8S rRNA molecule and three regions of spacer sequence. Initiation normally occurs at the beginning of the external transcribed spacer, although Rungger et al (1979) have shown that some initiation occurs at the partially reduplicated promoter region (Moss and Birnstiel, 1979) located within the non-transcribed spacer. The gene repeat therefore contains about 68.6% transcribed DNA, of which 76% (or 52% of the entire repeat) codes for the 18S and 28S rRNA molecules.

That the RNA made by the oocyte-treated nuclei is a correct rRNA transcript can be shown in a number of ways. The two chosen here are hybridization to a recombinant DNA probe and fractionation by sucrose gradients. Both of these have advantages and disadvantages when used with this system, a major problem that being the oocyte-treated nuclei synthesize only a very little RNA. However, this can be maximized by incubating the nuclei for longer and with increased amounts of ³H-UTP present in the assay. (15 μ Ci ³H-UTP/100 μ l assay) There is also a problem that cold rRNA is present in the oocyte extract; this can be minimized by using an extract prepared with a high speed spin (100,000g), and by spinning out



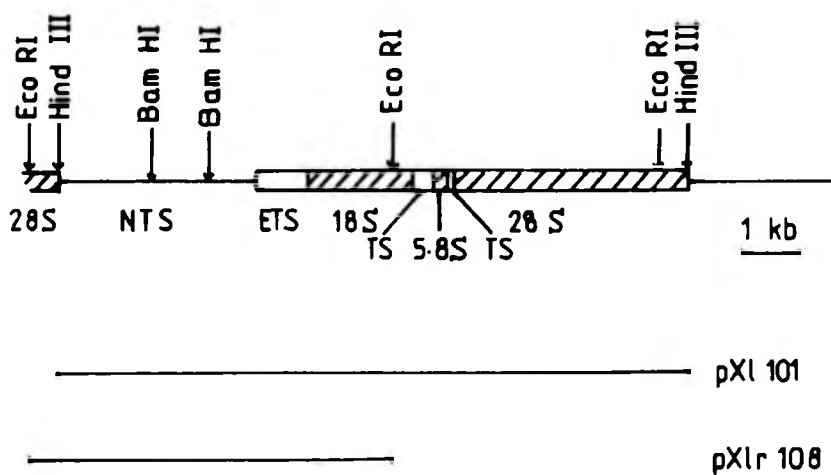


FIGURE 6.1

Restriction Map of the Ribosomal Gene Repeat of *Xenopus laevis*.

Data from Boseley et al (1978, 1979)

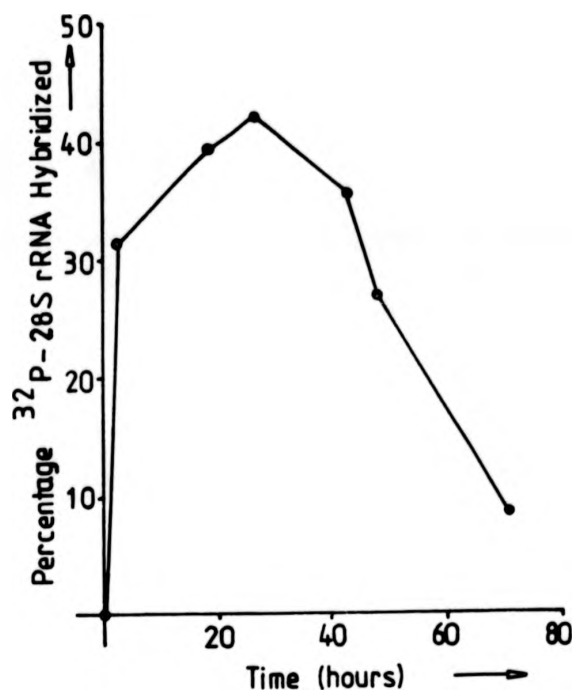


FIGURE 6.2

Optimal Time for RNA-DNA Hybridization.

Duplicate millipore filters loaded with 1 μ g of pX1101 DNA were placed in the bottom of 14 scintillation vial inserts and 100 μ l of hybridization buffer (50% deionized formamide, 3xSSC) and probe (32 P-28S rRNA at 6.4×10^5 cpm/ μ g, 8.6 ng/vial) added. These were incubated at 37°C for increasing times. Hybridized filters were washed in four washes of 2xSSC, dried and counted when all the filters had been processed.

TABLE 6.1 Presence of rRNA Transcripts from Erythrocyte Nuclei
Preincubated with Various Extracts.

RNA Source	Competing RNA	cpm binding	% Eff.	Internal Standards			
				³² P-18S RNA	³² P-28S RNA		
Nuclei +				cpm	% Eff.	cpm	% Eff.
oocyte extract	--	18510	16.36	524	19.9	397	20.1
oocyte extract	18S	11806	11.7	19	0.7	373	18.9
oocyte extract	28S	10103	10.2	507	19.25	7	0.35
oocyte extract	18S+28S	4255	4.2	12	0.46	9	0.46
neurula extract	--	29377	17.9	530	20.1	369	18.7
neurula extract	18S	18828	11.5	9	0.34	391	19.8
neurula extract	28S	17662	10.8	511	19.4	3	0.15
neurula extract	18S+28S	7105	4.3	16	0.6	5	0.25
egg extract	--	23619	19.6	561	21.3	401	20.3
+ Fr-18							
egg extract	18S	15146	12.6	20	0.75	409	20.7
+ Fr-18							
egg extract	28S	14173	11.8	531	20.2	9	0.46
+ Fr-18							
egg extract	18S+28S	5670	4.7	17	0.65	7	0.35
+ Fr-18							

1 μ g aliquots of pX1101 DNA was loaded onto Millipore filters and hybridized to the ³H-RNA samples listed above. The conditions were the same as outlined in Figure 6.2. Competing 18S and 28S rRNA was at a final concentration of 50 μ g/ml,

this decreases the hybridization of the 18S and 28S P-rRNA to less than 2%.

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+ Fr-18							
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this decreases the hybridization of the 18S and 28S P-rRNA to less than 2%.

the nuclei after the preincubation and resuspending them in extract-free buffer for the incubation. Sucrose gradients have high capacity, thus minimizing any over-loading problem, but their resolution is not as high as gel electrophoresis. Hybridization analysis has the dual advantages of high sensitivity and lack of dependence on the integrity of the molecules synthesized. The latter is important if ribonuclease is present or if elongation is inefficient. However, any cold rRNA present will decrease the efficiency of the hybridization.

Hybridization to the Ribosomal RNA Probe, pX1101.

The analyses were conducted on erythrocyte nuclei incubated with oocyte extract, neurula extract and egg extract plus Fr-18. α -amanitin (100 μ g/ml) was present to ensure that only RNA polymerase I was active. The three 3 H-RNA samples were hybridized to recombinant plasmid pX1101 which contains the entire ribosomal gene repeat [FIG 6.1]. The hybridization conditions were optimized using kinase-labeled 32 P-28S rRNA. The filter bound pX1101 and labelled rRNA were incubated for 24 hours to achieve maximum hybridization [FIG 6.2]. After this time the efficiency dropped from 40% as the filters became more fragile. 32 P-18S and 28S rRNA were used as internal standards in the subsequent hybridizations.

When the hybridizations are carried out [TABLE 6.1] using the 3 H-RNA transcribed by erythrocyte nuclei, the efficiency of the system is much less, as judged by the hybridization of the 32 P-rRNA. This reflects the continuing presence of contaminating cold rRNA in the samples, but since the efficiency is still quite high (20%), the amount of cold rRNA is small. In all cases, the

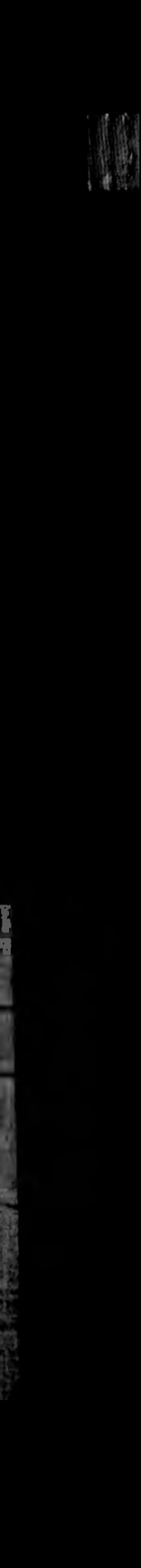


TABLE 6.2

Amount of Transcription of the 16S and 28S Regions by Treated Erythrocyte Nuclei.

RNA Source	% binding	cpm binding to		cpm not
nuclei +	to rDNA	18S region	28S region	competed off
oocyte extract	92.25	6704	8407	4255
neurula extract	89.1	10549	11715	7105
egg extract	92.0	9446	8473	5670
plus Fr-18				

³H-RNA from the treated erythrocyte nuclei binds to the probe just as efficiently as the internal standard ³²P-rRNA. So at least 92.25% of the RNA produced by oocyte-treated erythrocyte nuclei assigned to RNA polymerase I is transcribed from the ribosomal genes; the efficiency is 89.1% in the case of neurula-treated nuclei and 92% for egg plus Fr-18 treated nuclei. This degree of binding of the ³H-RNA from the nuclei to the ribosomal probe is reduced by the addition of cold 18S and 28S rRNA. If these are added in amounts sufficient to reduce the binding of the internal standard to zero the binding of the H-RNA is likewise reduced, but not to zero. In all three cases, some binding is still detectable. This (22.9% in the case of the oocyte-treated nuclei RNA or 18.4% of the pX1101 binding RNA) could reflect the presence of sequences transcribed from non-ribosomal DNA, the wrong strand of rDNA, or from its non-transcribed region, or as will be shown, the presence of transcribed spacer rRNA. The transcribed spacer accounts for 24% of the 40S precursor, and will only be present in trace amounts in the competing 18S and 28S rRNA. When competing 18S or 28S rRNAs are used alone, it can be seen that the 18S competes off slightly more and the 28S rRNA slightly less than might be expected from their size. The 18S rRNA sequence contributes 23.9% and the 28S rRNA sequence 52% of the precursor but they compete off 36.2% and 45.4% of the H-RNA from oocyte-treated nuclei respectively [TABLE 6.2]. For RNA isolated from nuclei preincubated with egg extract and Fr-18 the 18S sequences contribute 40% and the 28S sequences contribute 35.9% of the RNA synthesized leaving 24.1% not competed off. For RNA from nuclei preincubated with a neurula extract the values are 35.9% 18S and 39.8% 28S with 24.3% not competed off by the cold rRNA. This would indicate the presence of unfinished transcripts in the ³H-RNA from oocyte-treated nuclei.

For all three sources of the ^3H -RNA about 90% of this RNA binds to the entire ribosomal gene repeat unit. This demonstrates that it is indeed the ribosomal genes that are being transcribed by the RNA polymerase I molecules. That 77% of the ^3H -RNA binding to pXl101 can be successfully competed off by cold 18S and 28S rRNA indicates that not only is the correct strand being transcribed but also that it is mainly the transcribed regions that are being transcribed. The 23% that is not competed off can be accounted for by the transcribed spacer sequences. To show that this is the case, the recombinant plasmid pXlrl08 was used [FIG 6.1; 6.3]. This contains both the beginning and the end of the 40S transcript plus the entire non-transcribed spacer.

Hybridization to Restriction Endonuclease Fragments of Ribosomal Plasmid, pXlrl08.

Double restriction enzyme digests were carried out on pXlrl08; Bam HI + Eco RI and Bam HI + Hind III. The former digest yields four fragments, the three as shown [FIG 6.3] plus the plasmid vector. The second digest also gives four fragments, but since Hind III cuts within the plasmid vector, the 490 bp and 3350 bp fragments shown are attached to plasmid DNA. Using these restrictions, two fragments from the non-transcribed spacer region were isolated as follows. The digested DNA was run on an agarose gel, blotted onto nitrocellulose (Southern, 1975) and the resulting filter cut into small pieces to coincide with the gel tracks and DNA bands. These pieces were hybridized to ^{32}P -pXl101 DNA to check on the location of the fragments before hybridization to ^3H -RNA from oocyte-treated and egg plus Fr-18 treated nuclei.

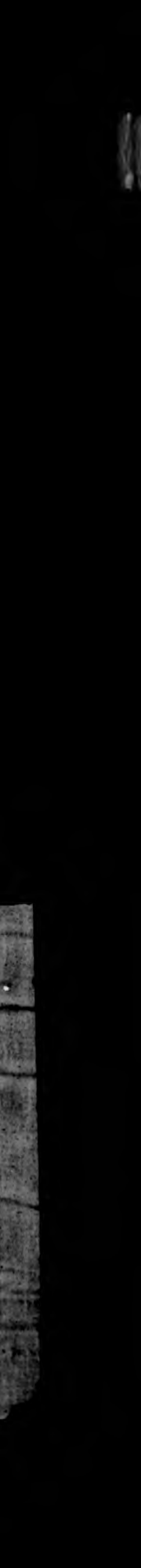


FIGURE 6.3

pXlr 108 \longleftrightarrow 500 bp

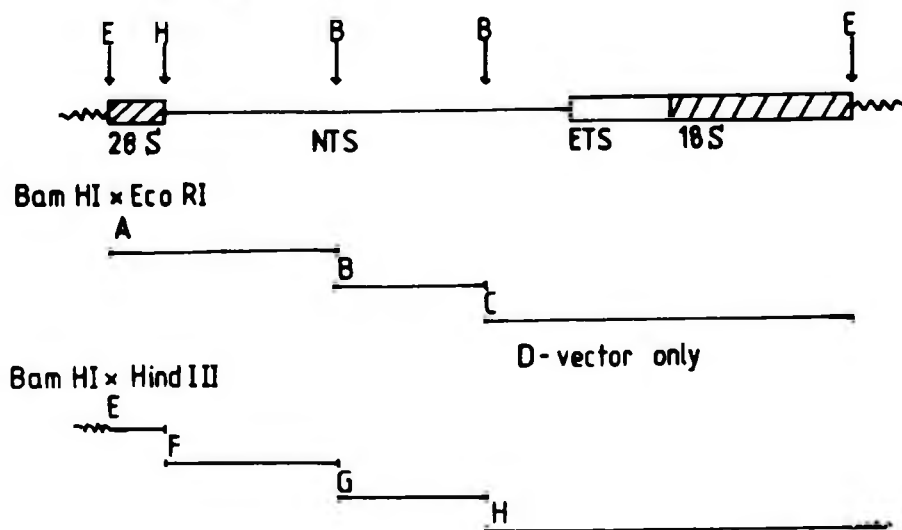


FIGURE 6.4

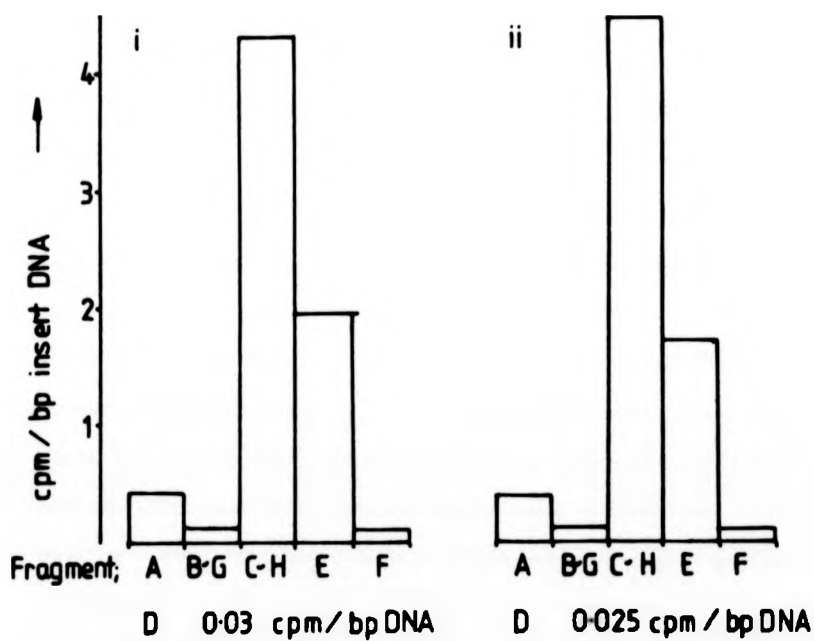


FIGURE 6.3

Restriction Map of rDNA Plasmid pXlr108.

Data from Boseley et al (1979)

FIGURE 6.4

Transcription of the rDNA Non-Transcribed Spacer in Erythrocyte Nuclei Pretreated with an Oocyte Extract.

The method is as outlined in the text. 5 µg of digested DNA being loaded onto the gel to be Southern blotted. The nitrocellulose pieces containing the relevant DNA fragments were hybridized for 24 hours (50% formamide, 3xSSC) with ³H-RNA isolated from erythrocyte nuclei preincubated with a S100 oocyte extract (i) or S100 egg extract plus Fr-18 (ii). After the 60 minute preincubation the nuclei were spun down and resuspended in the normal incubation mix for a further 60 minutes. The cpm bound to each filter is represented as cpm H-RNA bound/bp rDNA in that fragment.

There is little hybridization to the fragments containing only non-transcribed spacer DNA [FIG 6.3]. Some might perhaps have been seen at the reduplicated promoter region around the two Bam HI sites in the non-transcribed spacer (Moss and Birnstiel, 1979), since some initiation at this site has been described in vivo (Rungger et al, 1979). However, the treated nuclei do not make much use of these promoters and very little binding above background is seen to either fragment, 0.14 cpm/bp of these fragments compared to 0.03 cpm/bp of vector DNA and 4.35 cpm/bp of coding sequences. Most binding is seen to the large 3350 bp fragment containing the external transcribed spacer and the beginning of the 18S gene. There is also some binding to the small (490 bp) fragment of the end of the 28S gene but this is at a lower level. The small amount of hybridization seen to this fragment is not due to the low binding of such a small fragment to the nitrocellulose, since in the Hind III + Bam HI digest the 490 bp are attached to several kb of plasmid and should therefore bind well to the filter. The lower than expected amount of binding is probably a reflection of the number of transcripts that do not complete their transcription. either by falling off the gene or by elongating so slowly that they never reach the end of the gene. Calculating from the hybridization data only 30% of the transcripts reach full length. Those that do, do not continue on into the non-transcribed spacer since there is no binding to the 1690 bp Hind III + Bam HI fragment that immediately follows the end of the 28S gene. Since there is not a convenient fragment, it cannot be said whether initiation is correct. Certainly, there is very little transcription in the 1100 bp Bam HI fragment which is the nearest fragment to the start of the external transcribed spacer, and this could be due to false initiation of the reduplicated promoters. This problem should be



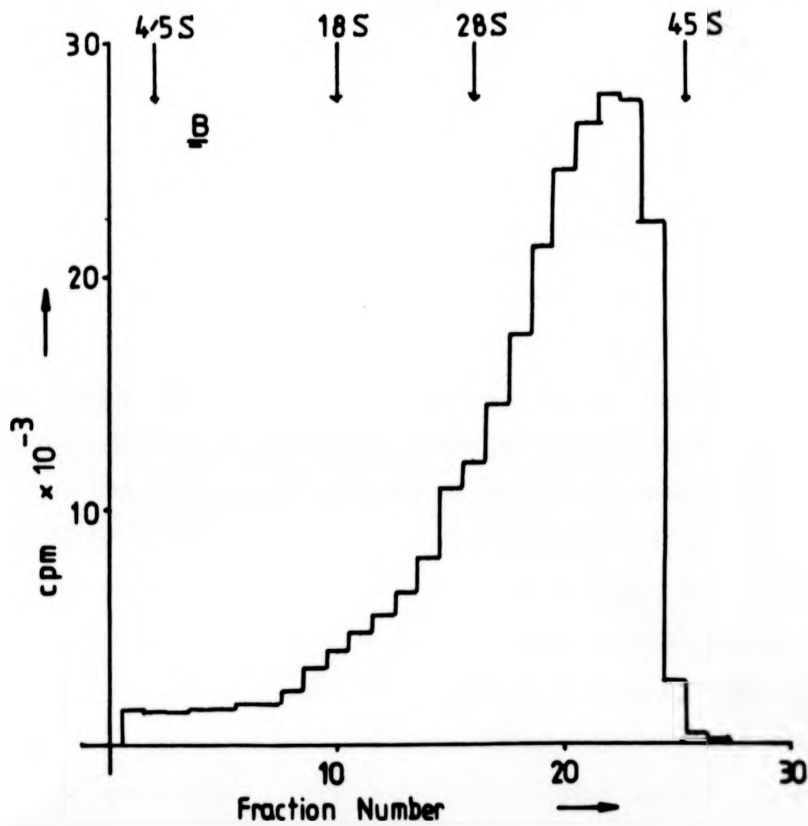
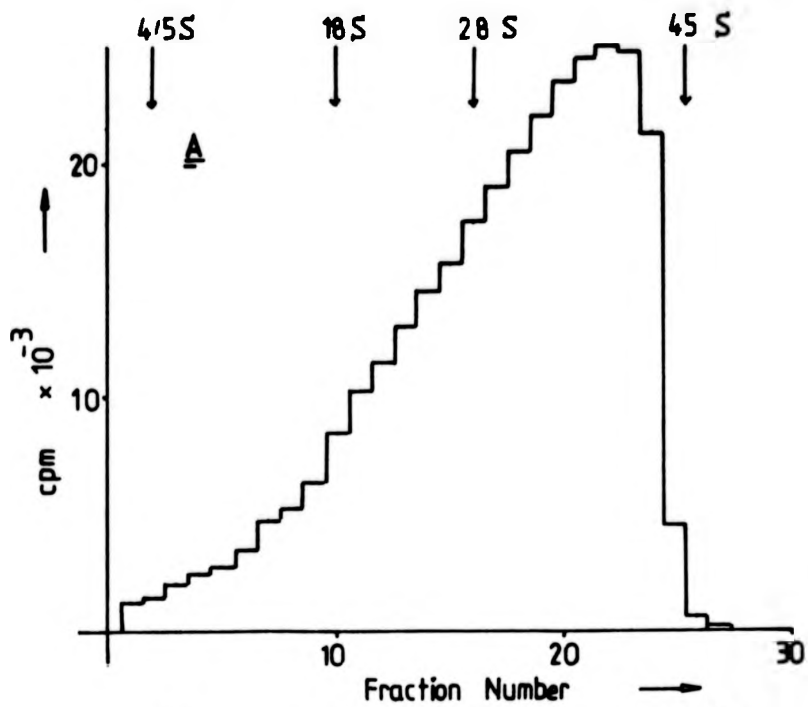


FIGURE 6.5

Sucrose Gradient Fractionation of ^3H -RNA Isolated from Erythrocyte Nuclei Preincubated with an Oocyte Extract.

^3H -RNA isolated from erythrocyte nuclei that had been preincubated with a S100 oocyte extract (A) or S100 egg extract and Fr-18 (B) was loaded onto 12 ml 5-20% sucrose gradients. These were centrifuged for 4 hours at 38,000 rpm at 4°C in the SW40 rotor of a Beckman L8 centrifuge. 400 μl fractions were taken, TCA precipitated and counted in a Packard Scintillation Counter. Parallel gradients were run with 4/5S, 18S, 28S and 45S RNA as markers, 18S and 28S RNA was added to the H-RNA samples immediately prior to loading to act as internal markers. These were located by measuring the OD at 260 nm of each fraction.

further investigated using S1 mapping.

Thus both oocyte-treated nuclei and nuclei preincubated with egg with Fr-18 and egg extract as the RNA polymerase source, yield RNA that by these hybridization criteria can be termed ribosomal RNA.

Fractionation of the RNA on Sucrose Gradients.

The ^3H -RNA produced by oocyte-treated nuclei does not yield a single high molecular band when run on a sucrose gradient [FIG 6.5], Instead a smear of radioactivity is seen throughout the gradient but with a sharp upper limit at about 41.5S. So a population of different sized H-RNA molecules are extracted from erythrocyte nuclei treated with an oocyte extract in the presence of α -amanitin, 92% of which hybridize to the ribosomal gene probe, and whose maximum size is 38 - 41.5S. Nuclei incubated with egg extract and Fr-18 plus 100 $\mu\text{g}/\text{ml}$ α -amanitin on the other hand, produce ^3H -RNA that has slightly less diversity in size, the upper limit is still the same but more of the RNA is of this size and less of the smaller size than the RNA from oocyte treated nuclei. Thus the RNA synthesized by both sorts of treated nuclei is of a variety of lengths. This variety is due to two factors; nucleases present in the protein extracts - oocyte extracts contain more of these and the RNA from oocyte-treated nuclei is of a more diverse size range - and to the slowness of elongation in this system. Under these conditions only 50.9% of the transcripts reach full length. This is calculated from the RNA made by nuclei treated with an egg extract plus Fr-18. It will still be a slight under estimate since the egg extract is not free from nucleases. The value for RNA isolated from nuclei

preincubated with an oocyte extract is 31.9%. No RNA transcripts were found above 41.5S which would indicate that initiation is correct, at least to the accuracy that can be measured here. Since termination is correct and the full length transcript is of the correct length, then initiation should occur at approximately the correct site as well.

The RNA polymerase I activity seen in erythrocyte nuclei treated with an oocyte extract is not mere non-specific transcription, but accurate transcription of the ribosomal genes. The protein semipurified from oocyte extracts by column chromatography also brings about accurate ribosomal RNA synthesis when added to an egg extract. A neurula extract has the same effect. In the erythrocyte nucleus, the oocyte component has an effect exclusively on the inert ribosomal genes; transcription by the other two RNA polymerases is not altered by Fr-18, it only permits accurate transcription by RNA polymerase I.

CHAPTER VII. THE DNASE I SENSITIVITY OF ERYTHROCYTE RIBOSOMAL DNA.

Since the initial discovery that the DNase I sensitivity of the globin genes in different cell types correlates with globin gene expression (Weintraub and Groudine, 1976), this relationship has been shown to hold true for a variety of genes. For example, the ovalbumin, conalbumin genes, both the α and β globin genes of chickens (Garel and Axel, 1976; Kuo et al., 1979; Stadler et al., 1980; Bellard et al., 1980), a number of heatshock protein genes in Drosophila (Wu, 1980; Keene et al., 1981) and the r-chromatin of Tetrahymena (Borchsenius et al., 1981). Several viral systems also follow this rule (eg Herbolat et al., 1981; Groudine et al., 1981). This sensitivity or resistance of a gene to DNase I has been taken as a reflection of the chromatin structure of that gene. It appears that an actively transcribed gene has a more open configuration, thus permitting easy access of DNase I molecules. A similar sensitivity is sometimes seen when micrococcal nuclease or DNase II are used (Reeves and Jones, 1976; Bloom and Anderson, 1978; Bellard et al., 1982; Larsen and Weintraub, 1982; Wood and Felsenfeld, 1982), although these enzymes probably recognise different facets of the same structure and subsequent digestion is also different.

The area of nuclease sensitivity encompasses not only the active gene itself, but may extend for several kb beyond. The DNase I sensitivity of the globin genes of chicken extends for 6-7 kb from the 5' end of the gene cluster and 8 kb from the 3' end (Stadler et al., 1980). Weisbrod and Weintraub (1981) have estimated the overall size of the DNase I sensitive region, or domain, to be 50-100 kb long, but within this large DNase I

sensitive domain are smaller domains of more acute DNase I sensitivity, corresponding to the region transcribed. The change from sensitive domain to resistant DNA is quite abrupt, and can be mapped (Weintraub et al., 1981). In several genes, the presence of sites exquisitely sensitive to DNase I have been shown (Kuo et al., 1979; Wu et al., 1979b; Wu, 1980; Keene et al., 1981). These do not normally occur within a gene, but are at a fixed location beside it. The distance between gene and DNase I 'hot spot' depends upon the system, it varies from 1 kb from the 5' end of the conalbumin gene (Kuo et al., 1979) to 300 bp upstream from the 5' end of many of the heat shock protein genes of Drosophila (Wu et al., 1979b; Wu, 1980; Keene et al., 1981). In some systems hypersensitive sites are present whatever the transcriptional state of the gene; for example the heat shock protein genes in Drosophila (Wu et al., 1979b; Wu, 1980; Keene et al., 1981) and the chicken conalbumin gene (Kuo et al., 1979) but in other cases these sites are only present in nuclei expressing these genes, eg the chicken embryonic α and U globin genes, certain of the sites associated with the Drosophila heat shock genes and the α and β globin genes (Stadler et al., 1980; Weintraub et al., 1981); the rat preproinsulin also conforms to this pattern (Wu and Gilbert, 1981).

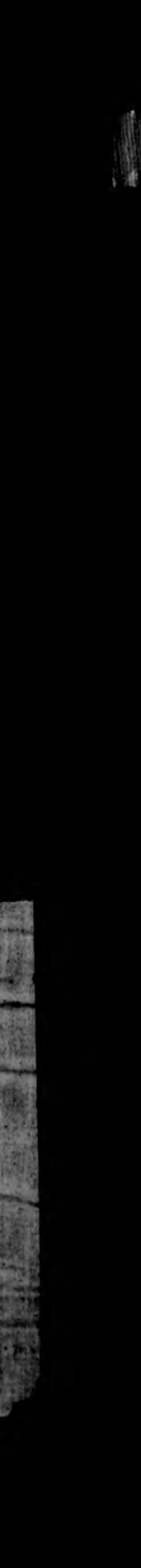
It has been suggested (Weintraub and Groudine, 1976; Young et al., 1978) that the change in chromatin configuration visualized by DNase I sensitivity of the gene, initially set up to allow transcription to proceed, is immovable and is not altered during subsequent divisions and further development of that cell. However, at least in the Drosophila heat shock

protein genes this is not so (Wu et al, 1979a). The change in transcription and DNase I sensitivity of these genes brought about by a heat shock is reversible. Once the cells are allowed to recover from the heat shock, the DNase I sensitivity, and presumably the chromatin configuration it reflects, returns to its pre-heat-shock state.

How the DNase I sensitive state is initiated in the first place and maintained is not known. The high mobility group (HMG) proteins, in particular HMG 14 and HMG 17, have been shown to be associated with the transcribed gene itself (Weisbrod and Weintraub, 1981) but not the entire, slightly DNase I sensitive domain. Although HMG 14 and 17 specifically bind to active genes, perhaps at the nucleosome level they do not make an inactive gene active (Weisbrod and Weintraub, 1981). Their role could therefore be in maintaining the active state rather than in initiating the change in chromatin configuration. The change in configuration is detectable, at least in respect of HMG 14 and 17 binding, at the nucleosome level, ie salt-washed nucleosomes from active genes that are depleted in non-histone proteins bind to HMG 14 and 17, whilst salt-washed nucleosomes containing

inactive sequences do not. However, no differences in protein and DNA content or physical properties have been seen between nucleosomes that bind and those that do not bind these HMG proteins (Weisbrod and Weintraub, 1981).

The ribosomal RNA genes of Xenopus laevis erythrocytes are not transcribed in the mature cell, although rRNA synthesis would have occurred at earlier developmental stages, nor are RNA polymerase I molecules present in the mature erythrocyte (Hentschell and Tata, 1978). These genes are therefore untranscribed and inactive and should be expected to be resistant



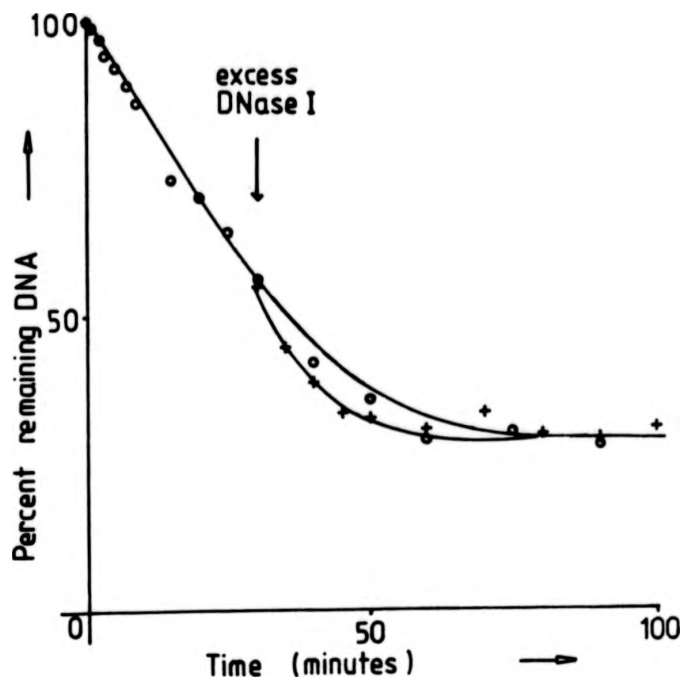


FIGURE 7.1

Digestion of Erythrocyte DNA by DNase I.

450 μ l of erythrocyte nuclei in RSB at a DNA concentration of 1 mg/ml were digested at 37°C with 20 μ g/ml DNase I (o---o). At times during this incubation a 25 μ l sample was removed, gently spun in an Eppendorf microfuge and the OD_{260} of the supernatant determined. A parallel assay (+---+) was carried out, starting at a DNase I concentration of 20 μ g/ml, but after 30 minutes this was increased to 50 μ g/ml.

to DNase I digestion.

Previous chapters show that I have identified a protein in a total oocyte extract, which brings about the transcription of the inactive ribosomal RNA genes of erythrocyte nuclei. To investigate whether this transcription of the erythrocyte rDNA is accompanied by an alteration in chromatin configuration, a series of DNaseI digestions and hybridization analyses were carried out to determine whether the ribosomal RNA genes of oocyte-treated nuclei were DNase I sensitive.

Effect of DNase I on Erythrocyte Nuclei.

Erythrocyte nuclei were incubated in RSB (Weintraub and Groudine, 1976) with DNase I for increasing lengths of time. To determine the incubation time required to achieve 5-10% digestion, the nuclei were spun out of the buffer and the absorbance at 260 nm of the supernatant measured [FIG 7.1]. About 30% of the DNA remains undigested despite the addition of excess DNase I. It takes 5 minutes for the DNase I to release 5-10% of the total DNA from the nuclei. This incubation time was used in all subsequent digestions.

Hybridization Conditions.

The conditions used were chosen in order to maximize the hybridization of the probe to the tested DNA - in this case ³H-pX1101 and total X.laevis DNA - whilst minimizing the non-specific binding of the probe DNA to the nitrocellulose filter. Thus PVP, Ficoll and non-competing bacterial RNA were used in a standard 50% formamide, 3xSSC, DNA/DNA hybridization at

Omitted from abstract



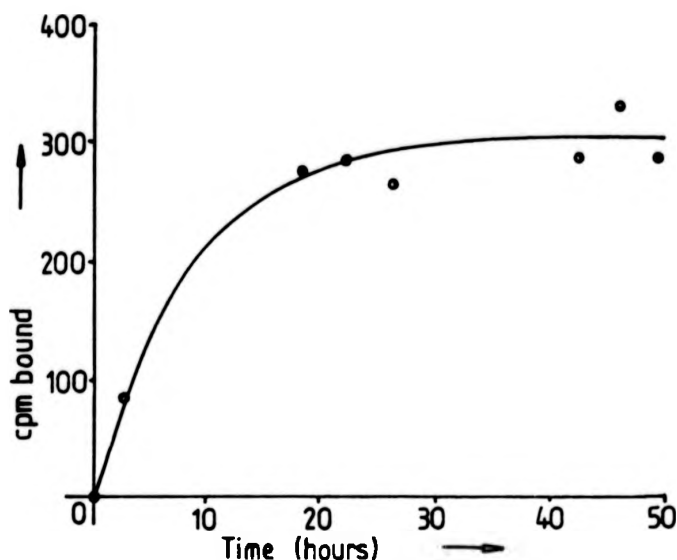


FIGURE 7.2

Optimum Time for DNA-DNA Hybridizations.

Eight vials were set up, each containing two identical nitrocellulose filters loaded with 1 μg total *X.laevis* DNA, Hybridization buffer (50% formamide pH 7.0, 3xSSC, 0.2% BSA, Ficoll, PVP and 0.25 mg/ml yeast tRNA) and 50 ng probe (^3H pX1101, 2.2×10^5 cpm/ μg) in final volume of 100 μl . These were incubated for up to 49.5 hours at 37°C and at times during this incubation a vial was removed and the filters washed (2xSSC, 3x30 minute washes) and dried. The filters were counted after the last time point in a Packard scintillation counter.

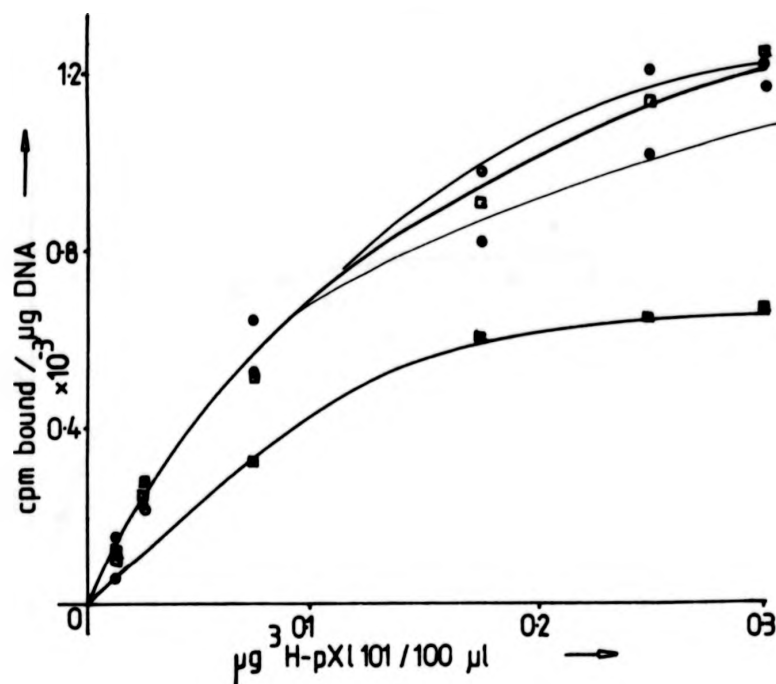


FIGURE 7.3

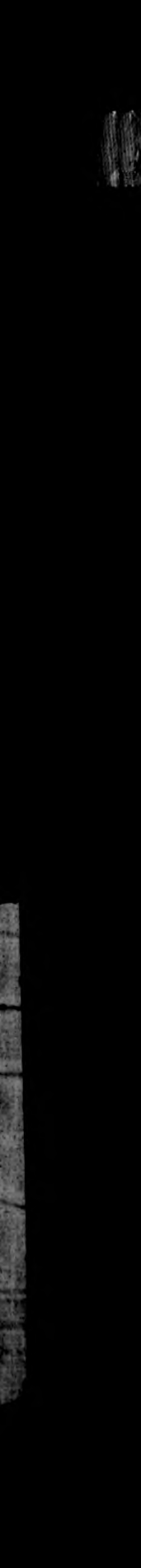
DNase I Sensitivity of the Ribosomal RNA genes in Erythrocyte and Liver Nuclei.

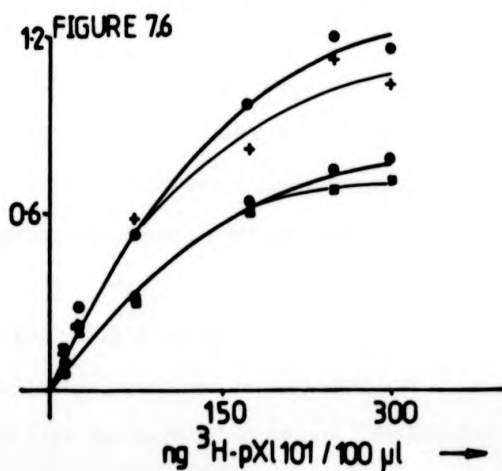
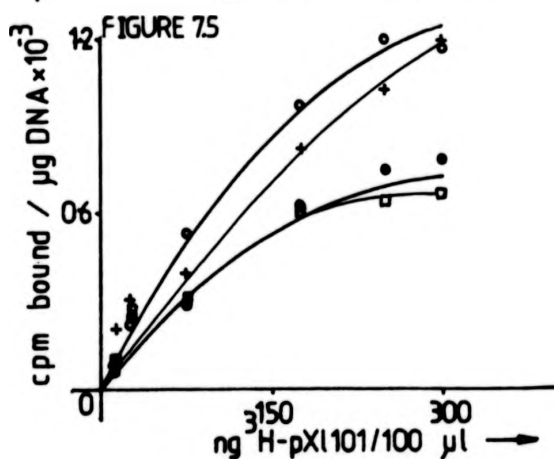
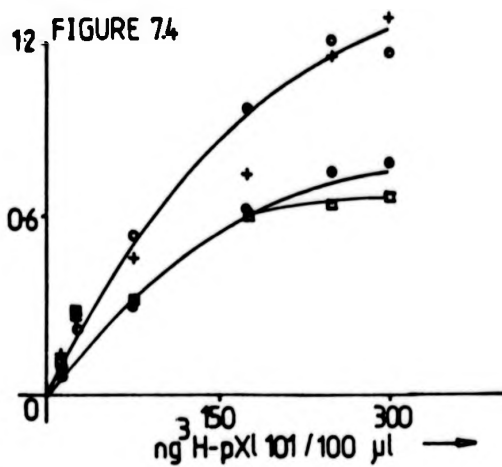
Nitrocellulose filters loaded with DNA from undigested liver (□---□) and erythrocyte (○---○) nuclei and DNase I digested liver (■---■) and erythrocyte (●---●) nuclei were hybridized for 24 hours with increasing amounts of $^3\text{H-pX1101}$ (2.2×10^5 cpm/ μg) in a volume of 100 μl of probe + buffer/2 duplicate filters. These were washed in 2xSSC after the hybridization, dried and counted. Results are expressed in this graph (and in Figures 7.4-7.6, 7.9, 7.11-7.14 and 7.16-7.23) as cpm bound/ μg nuclear DNA.

37°C. Denatured X.laevis DNA was loaded onto nitrocellulose filters and hybridized under these conditions to denatured, nick-translated ^3H -pX1101. This probe contains a single copy of the ribosomal RNA gene repeat. The hybridization was allowed to continue for 24 hours, which gives the highest amount of hybridization [FIG 7.2], before being washed and counted in a Packard Scintillation counter.

DNase I Sensitivity of the Ribosomal RNA Genes in Liver and Erythrocyte Nuclei.

Erythrocyte nuclei exhibit no rRNA synthesis, their RNA polymerase I molecules were lost before maturation and almost all of the transcription in vitro seen is as a result of the slow elongation of the final RNA polymerase II transcripts (Hentschell and Tata, 1978). Liver nuclei, on the other hand, are actively transcribing their ribosomal genes; 30.1% of their total transcription is as a result of RNA polymerase I activity [FIG 3.26]. So it is not surprising that the rDNA from liver nuclei was more sensitive to DNase I digestion than bulk DNA, but equally resistant in erythrocyte nuclei [FIG 7.3]. Both types of nuclei hybridized ^3H -pX1101 equally well after incubation in the absence of DNase I, indicating that the reduced hybridization to rDNA seen when liver nuclei are digested with DNase I, is not as a result of a nuclease present in the nuclear preparation.





FIGURES 7.4, 7.5, 7.6

DNase I Sensitivity of the rDNA in the Erythrocyte Nuclei
Preincubated with an Oocyte or Egg Extract.

Figure 7.4

Erythrocyte nuclei were preincubated in the normal way with an oocyte (●---●) or egg (+---+) extract. After 60 minutes the nuclei were spun down, washed with 0.5xRB and taken up at a DNA concentration of 1 mg/ml in RSB. These were DNased and the DNA extracted and loaded onto nitrocellulose filters in the normal way. Hybridizations were carried out as described in Figures 7.2 and 7.3 (probe ^3H -pX1101 at 2.2×10^5 cpm/ μg). As controls, total DNA (o---o) and DNA from DNased liver nuclei (□---□) were also hybridized in the same way.

Figure 7.5

Erythrocyte nuclei were preincubated in the normal way with an oocyte extract, washed with 0.5xRB and taken up in RSB at 1 mg DNA/ml. The nuclei were either incubated with (●---●) or without DNase I (+---+), before the DNA was extracted. Hybridizations were carried out as described previously (probe ^3H -pX1101 at 2.2×10^5 cpm/ μg). (o---o) total DNA; (□---□) DNA from DNased liver nuclei.

Figure 7.6

Erythrocyte nuclei were preincubated in the normal way with egg extract plus Fr-18, washed with 0.5xRB and taken up in RSB at 1 mg DNA/ml. The nuclei were either incubated with (□---□) or without DNase I (+---+), before the DNA was extracted. Hybridizations were carried out as described previously (probe ^3H -pX1101 at 2.2×10^5 cpm/ μg). (o---o) total DNA; (●---●) DNA from oocyte treated DNased erythrocyte nuclei.

It is possible to induce erythrocyte nuclei to synthesize rRNA by preincubating them with an oocyte extract. Nuclei preincubated in this way were incubated with DNase I such that 5-10% of their DNA was solubilized, and the remainder was extracted. Nuclei preincubated with an egg extract were also treated with DNase I. Pre-treatment with an egg extract has no effect on the DNase I sensitivity of the rDNA in erythrocyte nuclei [FIG 7.4], whereas pre-treatment with an oocyte extract does. In such nuclei, the ribosomal genes are DNase I-sensitive, although not quite as sensitive as the rDNA of liver nuclei.

This increase in DNase I sensitivity in oocyte-treated nuclei is not a reflection of any nucleases that are present in the oocyte extract itself. When the DNA from nuclei preincubated with an oocyte extract was extracted without the DNase I step, no increased sensitivity of the rDNA is seen [FIG 7.5].

Egg-treated erythrocyte nuclei can also be induced to transcribe rRNA if Fr-18 is present during the preincubation. Nuclei preincubated in this way also show an increased sensitivity of their rDNA to DNase I digestion [FIG 7.6]. The degree of sensitivity is similar to that seen in oocyte-treated nuclei. It is not brought about by specific nucleases within the column fraction since if the nuclei are not treated with DNase I after the preincubation, the DNA extracted does not show this reduced rDNA content.

Thus the appearance of transcription by RNA polymerase I

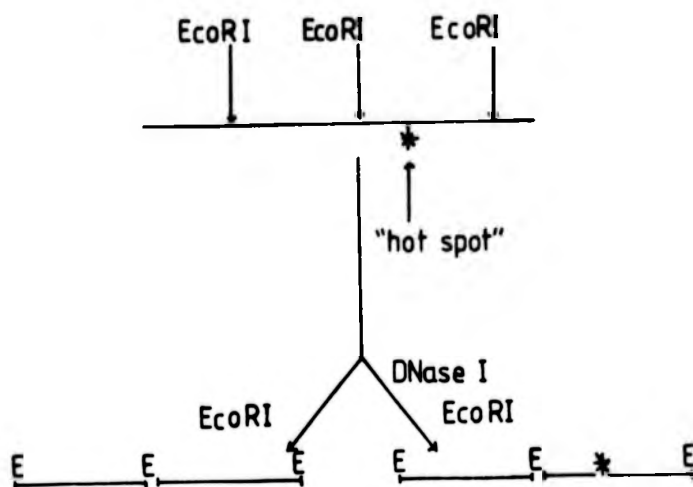
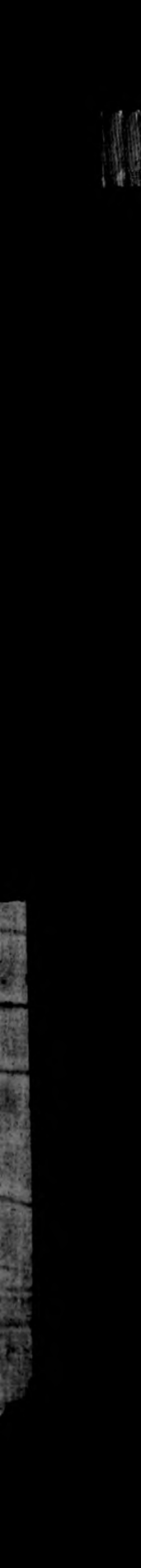


FIGURE 7.7

Effect of a DNase I Hypersensitive Site.

when erythrocyte nuclei are treated with an oocyte extract or an egg extract plus Fr-18, is mirrored in the chromatin structure. The rDNA becomes more open and accessible to DNase I as a result of these treatments. Whether the whole gene or just part of it is sensitive, can be determined by a variation on the above hybridization method. If the DNase I-treated nuclear DNA is digested with a restriction enzyme and run out on an agarose gel, blotted onto a nitrocellulose filter (Southern, 1975) and then probed with a ^{32}P -probe, the bands seen on the autoradiogram of the washed filter will depend on how many times the particular restriction enzyme cuts the gene being probed for. For instance, an Eco RI restriction digest of total Xenopus laevis DNA, probed by ^{32}P -pXl101 will reveal two bands of about 4.8 kb and 6.63 kb representing the two Eco RI sites within the rRNA gene. If on repeating with DNA from DNase I digested, oocyte extract-treated nuclei, one band is fainter than the other, once proper account is taken of the size difference between the fragments, it can be said that the fainter fragment is preferentially digested by DNase I. That is, it is in a more open configuration than the other part of the gene. The presence of a hypersensitive site (Wu et al., 1979a) of acute DNase I sensitivity would be seen by an increase in the number of bands present or a decrease in size of a band [FIG 7.7], since a "hot spot", under this analysis acts as an extra restriction site. Thus the simple hybridization analysis used here measured the rate at which a given region is digested to non-hybridizable fragments. The blot method, outlined above, reflects the possibility of introducing the first nuclease cut into a fragment which may, or may not, be homogeneously more accessible to the DNase I molecules. Therefore, a genomic region which is more rapidly converted to non-hybridizable fragments must also be more sensitive using the blot hybridization assay,



A B C



FIGURE 7.8

Presence or Absence of an Hypersensitive Site in rDNA.

Erythrocyte nuclei in RSB at 1mg DNA/ml were digested with DNase I such that only 0.5 - 1% of the DNA is lost, after the digestion the incubations were made 12.5 mM EDTA, 0.5% SDS and the DNA extraction procedure carried on from this point. The DNA from nuclei preincubated with buffer (Track B) and oocyte extract prior to DNase I digestion (Track C) as well as total DNA (Track A) was digested with Eco RI, run on a 1% agarose gel (2 μ g digested DNA/slot) and transferred to a nitrocellulose filter (Southern, 1975) which was probed with 32 P-pX1101 (2×10^7 cpm/ μ g).



FIGURE 7.8

Presence or Absence of an Hypersensitive Site in rDNA.

Erythrocyte nuclei in RSB at 1mg DNA/ml were digested with DNase I such that only 0.5 - 1% of the DNA is lost, after the digestion the incubations were made 12.5 mM EDTA, 0.5% SDS and the DNA extraction procedure carried on from this point. The DNA from nuclei preincubated with buffer (Track B) and oocyte extract prior to DNase I digestion (Track C) as well as total DNA (Track A) was digested with Eco RI, run on a 1% agarose gel (2 μ g digested DNA/slot) and transferred to a nitrocellulose filter (Southern, 1975) which was probed with 32 P-pX1101 (2×10^7 cpm/ μ g).



FIGURE 7.6

Presence or Absence of an Hypersensitive Site in rRNA.

Erythrocyte nuclei in K80 at 1mg RNA/ml were digested with DNase I such that only 0.5 - 1% of the RNA is lost; after the digestion the incubations were made 12.5 mM EDTA, 0.5% SDS and the RNA extraction procedure carried on from this point. The RNA from nuclei preincubated with buffer (Track B) and onocyte extract prior to DNase I digestion (Track C) as well as total RNA (Track A) was digested with 32 P-labeled, run on an 8% agarose gel (2nd undigested RNA slot) and transferred to a nitrocellulose filter (Southern, 1975) which was probed with 32 P-X1101 (2x10⁶ cpm/ug).

A B C



FIGURE 7.8

Presence or Absence of an Hypersensitive Site in rDNA.

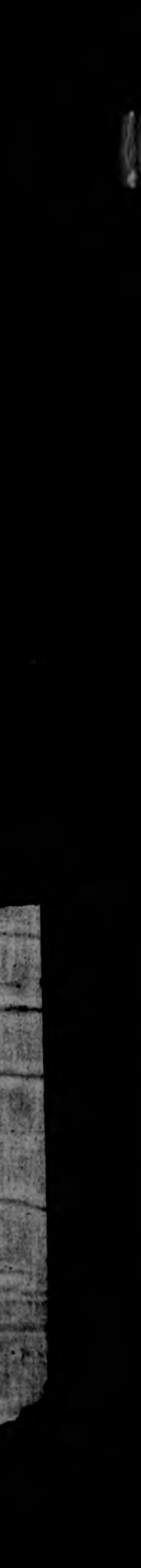
Erythrocyte nuclei in KSB at 1mg DNA/ml were digested with DNase I such that only 0.5 - 1% of the DNA is lost, after the digestion the incubations were made 12.5 mM EDTA, 0.5% SDS and the DNA extraction procedure carried on from this point. The DNA from nuclei preincubated with buffer (Track B) and oocyte extract prior to DNase I digestion (Track C) as well as total DNA (Track A) was digested with Eco RI, run on a 1% agarose gel (2 μ g digested DNA/slot) and transferred to a nitrocellulose filter (Southern, 1975) which was probed with P-X1101 (2×10^7 cpm/ μ g).

but the reciprocal is not necessarily true. For example, the presence of specific DNase I cleavage points also results in the disappearance of a given restriction fragment. So whilst the procedure used standardly here will not detect a hypersensitive site in the way that a blot-transfer hybridization of electrophoresed DNA will, it does give a more accurate quantitative assay of the DNase I sensitivity of the gene as a whole.

To determine whether there is a hypersensitive site associated with the ribosomal genes, and to see if one part of the gene is more sensitive to DNase I digestion than the rest, the method outlined above was carried out. There is no hypersensitive site associated with the ribosomal genes from either buffer or oocyte-treated nuclei [FIG 7.8]; neither is one part of the gene digested preferentially by DNase I - all of the gene is equally sensitive, both transcribed and non-transcribed sequences.

The above changes in DNase I sensitivity of the ribosomal genes were all seen in nuclei where transcription was occurring. It could be that it is transcription per se that causes the DNase I sensitivity and not the change to a more open chromatin configuration that allows the RNA polymerase molecules access to the gene. However, this would not explain why the non-transcribed spacer region is as sensitive as the transcribed region of the rRNA genes in oocyte-treated nuclei, or why large domains of sensitive DNA have been shown to extend for several kb around the transcribed gene sequence (Weisbrod and Weintraub, 1981).

Erythrocyte nuclei were preincubated in such a way that



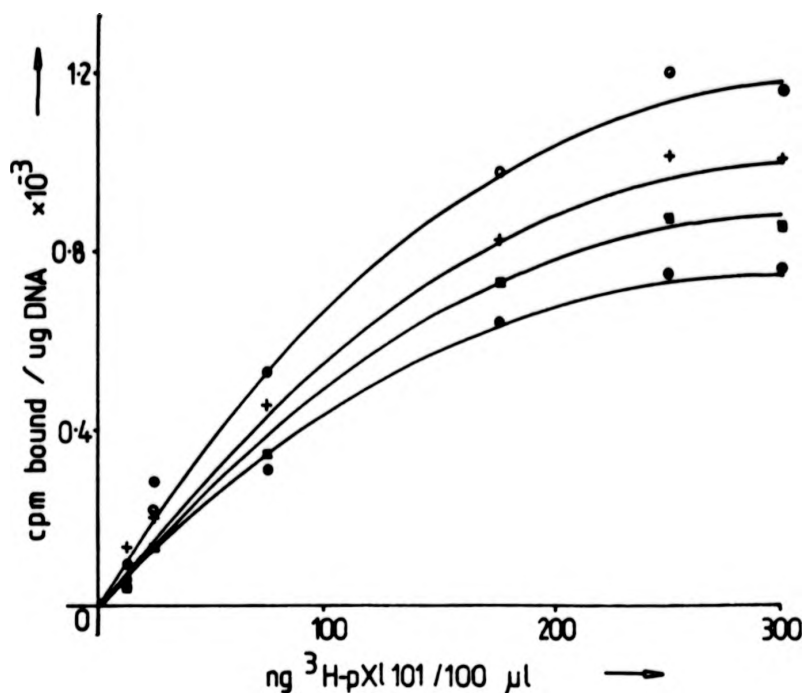


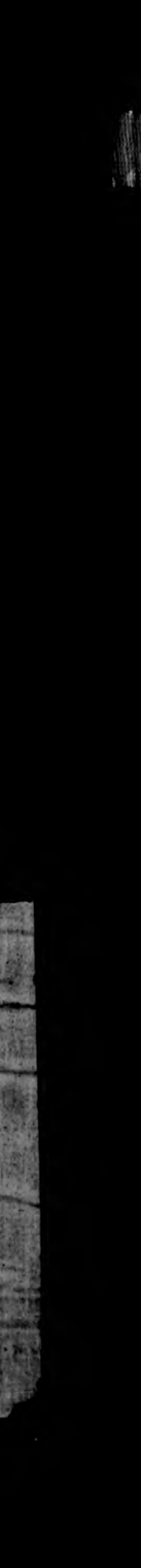
FIGURE 7.9

DNase I Sensitivity of rDNA in Preincubated Erythrocyte Nuclei where Transcription was Inhibited.

Erythrocyte nuclei were preincubated for 60 minutes with an oocyte extract and 100 $\mu\text{g}/\text{ml}$ Actinomycin D ($\square\text{---}\square$) or Fr-18 alone ($+\text{---}+$) before being washed in $0.5\times\text{RB}$, digested with DNase I and the DNA extracted. Hybridizations were carried out as previously described (probe $^3\text{H-pXl101}$ at 2.2×10^5 cpm/ μg), with total DNA ($\circ\text{---}\circ$) and DNA from erythrocyte nuclei preincubated with an oocyte extract prior to DNase I digestion ($\bullet\text{---}\bullet$) also included.

none, or very little, transcription of the ribosomal genes could take place. When erythrocyte nuclei are preincubated with Fr-18 alone, no transcription can be measured over that seen in control nuclei, since RNA polymerase I is absent. By omitting a RNA polymerase source, the effect of Fr-18 by itself on the rDNA can be seen. If RNA polymerase I molecules are necessary to induce DNase I sensitivity - although the effect on leaving them out of the preincubation and adding them at the beginning of the transcription assay [TABLE 5.6] would indicate that they are not - no increased sensitivity of the rDNA should be seen. Erythrocyte nuclei were also preincubated with an oocyte extract plus actinomycin D. Although this will not prevent the initial binding of the RNA polymerase I molecules on the genes, it will prevent further elongation (Widnell and Tata, 1966; TABLE 3.5).

When the erythrocyte nuclei were preincubated with only Fr-18, there was an increase in the DNase I sensitivity of the rDNA but not to the same extent as seen in oocyte treated nuclei [FIG 7.9]. The nuclei preincubated with an oocyte extract plus actinomycin D also showed that their rDNA was more sensitive to DNase I, but it was not as sensitive as when actinomycin was absent, but more so than if only Fr-18 was added. This difference between the sensitivities of the rDNA in these variously treated nuclei could be explained in one of the two following ways. Either only a few rRNA gene repeats are sensitized in nuclei pre-treated with Fr-18, and more when the nuclei are pre-treated with oocyte extract plus actinomycin D; or only part of the rRNA gene is made accessible to DNase I in nuclei preincubated with Fr-18, and more of the gene repeat is opened up and accessible in the nuclei preincubated with oocyte extract and Actinomycin D. Results given above show that the whole gene repeat is sensitive



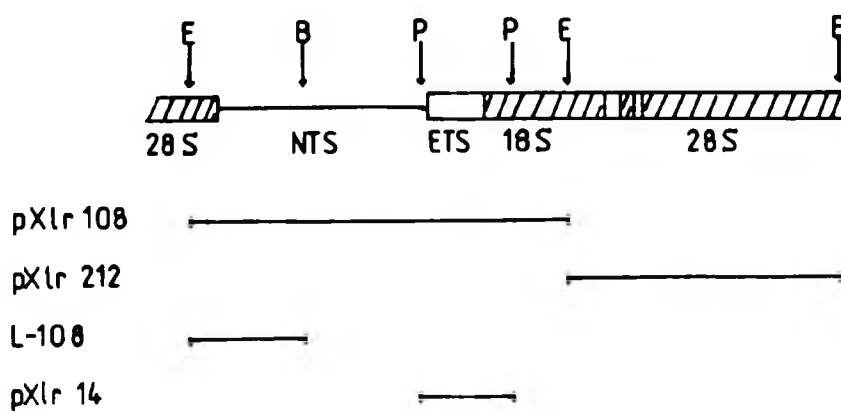


FIGURE 7.10

Restriction Map of *Xenopus laevis* Ribosomal DNA.

Data from Boseley et al (1978,1979).

E - Eco RI

B - Bam HI

P - Pst I

to DNase I in nuclei preincubated with an oocyte extract alone.

In order to determine which of these two possibilities is the correct one, a series of ribosomal gene fragments were used as probes [FIG 7.10]. pXlr108 and pXlr212 are the two Eco RI fragments of the ribosomal gene repeat. pXlr108 contains all of the non-transcribed spacer flanked by the last 490 bp of the 28S gene and the first 2.52 kb of the 40S transcript; the latter includes all of the external transcribed spacer. pXlr212 only contains transcribed sequence, it completes the gene repeat with the central part of the 40S precursor. L-108 is a 2.18 kb fragment cut from pXlr108 and contains the final 490 bp of the 28S gene and 1.69 kb of the non-transcribed spacer. pXlr14 is a subclone of pXlr108 containing a 1.57 kb Pst I fragment encompassing all of the external transcribed spacer and the first 600 bp or so of the 18S gene sequence, it contains no non-transcribed spacer. If the difference between the rDNA from nuclei treated with Fr-18 and oocyte extract is just a reflection of the numbers of ribosomal genes fully accessible to DNase I, then all of the above probes will give the same degree of hybridization as seen in Fig 7.9, in which pXlr101 was the probe. If, on the other hand, this amount of hybridization is a reflection of the different degrees of openness of the ribosomal genes, different probes will hybridize to differing extents depending on whether they cover the DNase I sensitive region or not.

When the rDNA of erythrocyte nuclei treated with oocyte extract in the presence of actinomycin D is probed with pXlr212 a high degree of sensitivity to DNase I is revealed [FIG 7.11]. This contrasts with the intermediate sensitivity seen when pXlr101

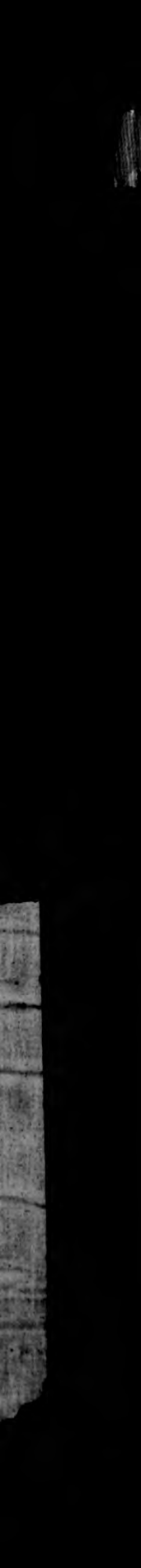


FIGURE 7.11

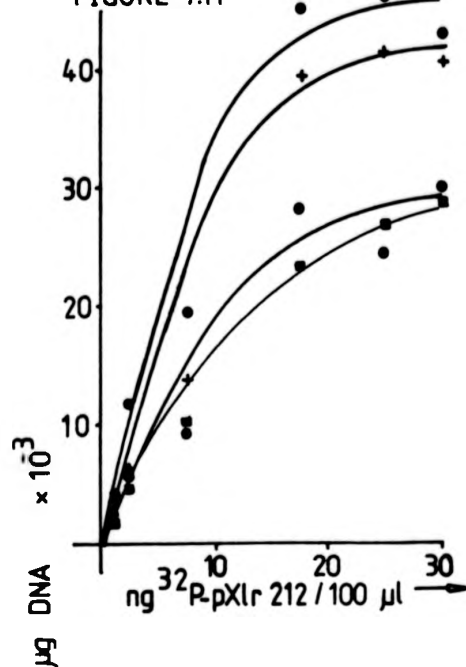


FIGURE 7.12

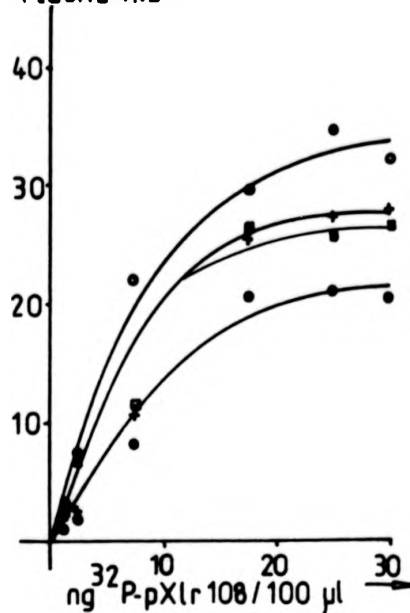


FIGURE 7.13

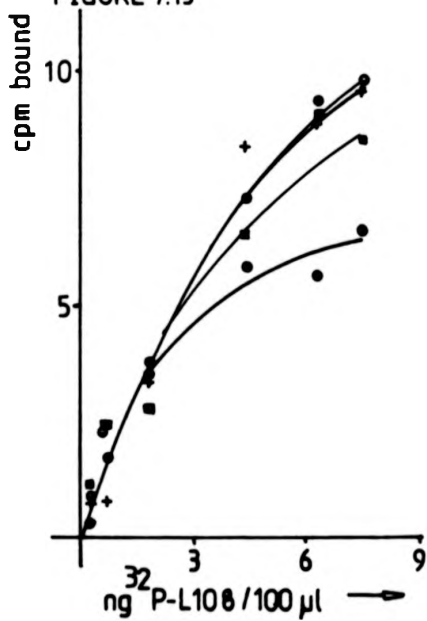
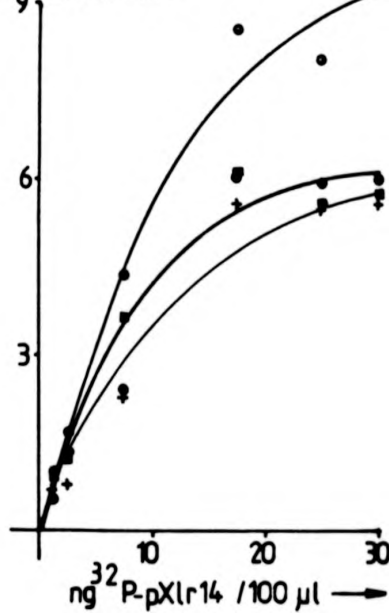


FIGURE 7.14



FIGURES 7.11 - 7.14

DNase I Sensitivity of Parts of the rRNA Gene in Erythrocyte Nuclei.

The DNAs described in Figure 7.9 were hybridized with the four rDNA probes shown in Figure 7.10 as described previously.

- (o---o) Total DNA
- (●---●) DNA from erythrocyte nuclei preincubated with an oocyte extract before DNase I digestion.
- (□---□) DNA from erythrocyte nuclei preincubated with an oocyte extract and Actinomycin D prior to DNase I digestion.
- (+---+) DNA from erythrocyte nuclei preincubated with Fr-18 only before DNase I digestion.

Figure 7.11 probe ^{32}P -pXlr212, specific activity 1.5×10^7 cpm/ μg

Figure 7.12 probe ^{32}P -pXlr108, specific activity 5.6×10^6 cpm/ μg

Figure 7.13 probe ^{32}P -L-108, specific activity 1.8×10^7 cpm/ μg

Figure 7.14 probe ^{32}P -pXlr14, specific activity 1.1×10^6 cpm/ μg

is the probe. In contrast Fr-18 alone does not make the portion of the ribosomal gene represented by pXlr212 as DNase I sensitive.

Probing with pXlr108 gives another pattern of hybridization [FIG 7.12]; rDNA from nuclei preincubated with Fr-18 is again slightly sensitive to DNase I digestion, but rDNA from nuclei pre-treated with an oocyte extract and actinomycin D is also only slightly sensitive to DNase I, although more so than the Fr-18 treated nuclei. In both cases, the degree of sensitivity is not as great as in rDNA from oocyte-treated nuclei. Thus in nuclei treated with actinomycin D plus oocyte extract, it seems that part of the ribosomal gene homologous to pXlr108 is much less open and accessible to DNase I digestion than that hybridizing to pXlr212.

When fragment L-108 was used as a probe [FIG 7.13], the rDNA from Fr-18 treated nuclei was shown to be insensitive to DNase I digestion; the amount of hybridization seen is comparable to that found using untreated DNase I digested nuclei. The same region in oocyte plus actinomycin D treated nuclei is only slightly sensitive to DNase I, whilst in oocyte treated nuclei it is as sensitive as any other region of the ribosomal gene. In comparison, probing with pXlr14, which contains the beginning of the transcribed region as opposed to its end [FIG 7.14], shows that this region is equally DNase I sensitive in all three types of treated nuclei.

Thus probing with these four ribosomal gene fragments suggests that the degree of hybridization seen in Figure 7.9 is a result of differences in the chromatin configuration of different

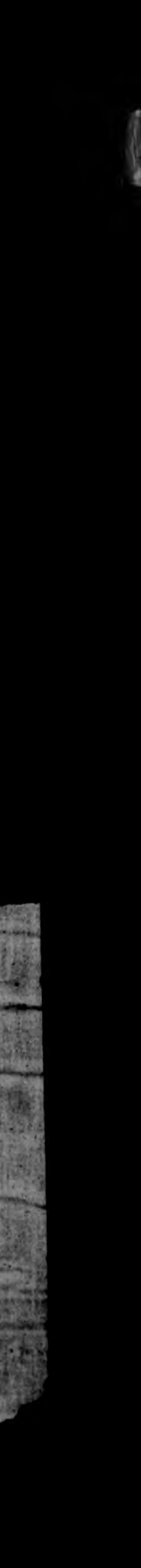


TABLE 7.1

DNase I Sensitivity of Regions of the Ribosomal RNA Gene
Repeat.

DNA Source; Erythrocyte nuclei preincubated with extracts prior to DNase I digestion;				
Probe	Total DNA	oocyte extract	oocyte extract + Actinomycin D	Fr-18
pXlr212	100	69.6	66.9	94.7
pXlr108	100	64.0	82.8	87.8
L-108	100	68.4	86.6	98.6
pXlr14	100	65.0	62.9	59.8

These data are calculated from the cpm bound/ μ g nuclear DNA at the maximum amount of probe used for each experiment as shown in Figures 7.11-7.14. They represent the percentage binding of the probe to the DNA assuming that the value for undigested DNA is 100%.

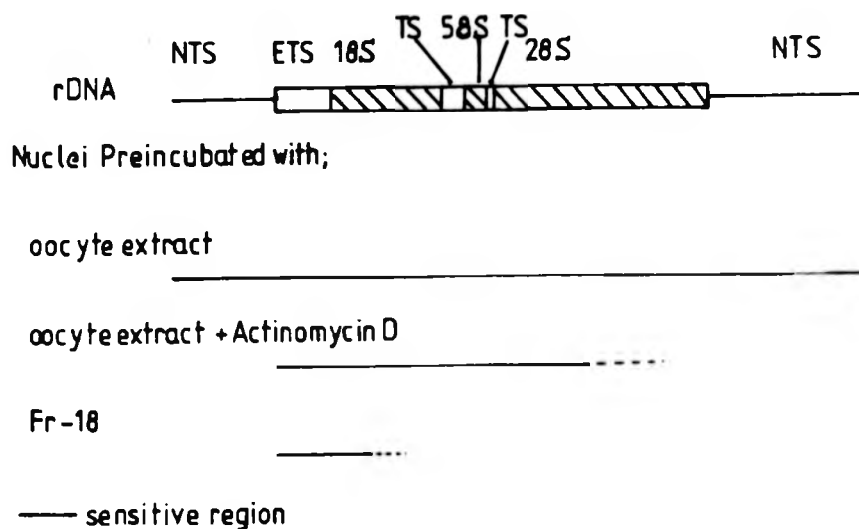


FIGURE 7.15

Differential DNase Sensitivity of the rDNA from Erythrocyte Nuclei.

Data for this figure comes from Figures 7.11 - 7.15.

regions within the ribosomal genes. In nuclei preincubated with an oocyte extract, all of the gene repeat is equally sensitive to DNase I [FIG 7.8; FIGS 7.11-14; TABLE 7.1], such that the 5% of the DNA lost by DNase I digestion contains about 35% of the ribosomal gene sequences. When transcription in these nuclei is prevented by the addition of actinomycin D, only the transcribed region is sensitive to DNase I and not the non-transcribed spacer sequence [FIG 7.15]. In such nuclei the addition of DNase I results in the loss of about 35% of the sequences represented by pXlr14 and pXlr212, whilst only 17.2% of pXlr108 sequence and 11.4% of L-108 sequence. The small, but measurable sensitivity can be accounted for by the transcribed sequence found in both pXlr14 and L-108, leaving the non-transcribed spacer DNase I resistant. Incubation of erythrocyte nuclei with Fr-18 alone results in only the first part of the ribosomal gene becoming DNase I sensitive. Thus 40.7% of the pXlr14 sequence is lost by DNase I digestion, but none (2.1%) of the L-108 sequence. The slight loss (12%) of the pXlr108 sequence is presumably because it contains the pXlr14 sequence within it. There is very little loss (5.8%) of pXlr212 sequence which probably represents the extreme 5' end of the sequence, ie that in the 28S region, being DNase I sensitive, whilst the rest of the sequence is resistant.

Fr-18 therefore acts, at least in part, by altering the conformation of the chromatin structure of the ribosomal genes. It does this without the presence of RNA polymerase I which transcribes these genes. The alteration in chromatin conformation to a more open and DNase I accessible form is limited to the beginning of the gene sequence coding for the 40S precursor [FIG 7.15]. The end of this region and the non-transcribed spacer are unaffected. The amount of DNase I sensitivity seen when pXlr108

is used as probe can be accounted for by the sensitivity of the beginning of the transcribed region contained within this probe. The reduplicated promoter region (Moss and Birnstiel, 1979) located within the non-transcribed spacer does not appear to react to Fr-18 and to become DNase I sensitive, ie Fr-18 appears to bind to a region in or near the external transcribed spacer that is not reduplicated. When RNA polymerase I is present, this area of more open chromatin structure is extended along the gene somewhat, however, it still does not extend into the non-transcribed spacer. The DNase I sensitivity of this region is only altered when the gene is being transcribed. This could, perhaps, simply be due to the bulky RNA polymerases and attached transcript that would exist either side of the non-transcribed spacer, forcing this region into a more open conformation. This does not explain why the newly initiated, but non-transcribing, RNA polymerase I molecules of the nuclei preincubated with an oocyte extract and actinomycin D cause the gene to become DNase I sensitive. These molecules are not as bulky as ones that are transcribing as they are not associated with the growing rRNA transcript, so their ability to have such a far reaching effect on the chromatin structure 7 kb away is uncertain. It does not explain either, why, if their physical presence is all that is needed for DNase I sensitivity to extend into the gene, this change in conformation does not extend in the opposite direction into the non-transcribed spacer. On the other hand the actinomycin D might not only be preventing RNA polymerase I elongation but also inhibiting some other component of the oocyte extract, for example a nicking-closing enzyme or gyrase that would, once initiated by the presence of the relevant component of Fr-18, open it out in a 5' to 3' direction relative to transcription. If a molecule like this is responsible for opening

up the ribosomal genes, it must be present in egg extracts, since Fr-18 plus egg extract also cause the entire ribosomal gene to become DNase I sensitive. Likewise it should also be present in the crude RNA polymerase preparation used in some of the transcription assays if the transcription seen in the oocyte column assays is paralleled by an increase in the DNaseI sensitivity of the ribosomal genes, since the crude RNA polymerase preparation can be used in place of an egg extract in the oocyte column transcription assay with identical results as regards transcription, albeit at a slightly lower level. Oocyte fraction 18 itself contains no nicking-closing enzyme or gyrase, and these by themselves - as seen by the addition of oocyte fractions 10-12 [FIG 5.6] - have no effect at all on transcription. So both would have to be present for the entire gene to become DNase I sensitive, Fr-18 directing the action of the gyrase or nicking-closing enzyme. Of course, some other entirely different mechanism might be responsible for this phenomenon.

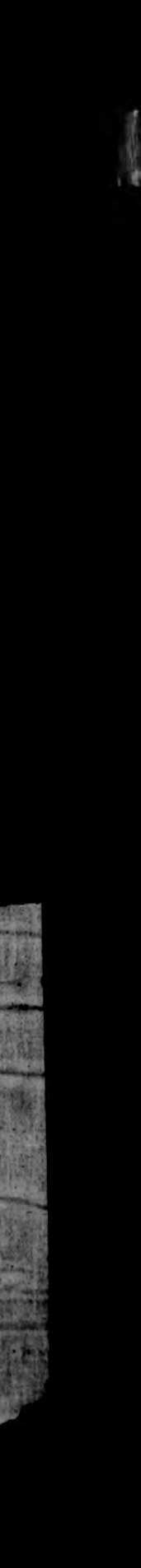
The control of ribosomal gene activity has been linked to the degree of methylation of the cytosine bases in the non-transcribed spacer (Bird et al., 1981). The onset of demethylation in two defined regions (Bird and Southern, 1978) has been correlated with the onset of ribosomal RNA synthesis during embryogenesis. This pattern of demethylation is inherited by all subsequent somatic cells, such that even erythrocytes, which do not synthesize rRNA, have these regions of demethylation in their ribosomal non-transcribed spacer sequences. However, the rRNA of the closely related X.borealis remains demethylated at these sites throughout its development and no methylation - demethylation cycle is seen, even though the pattern of rRNA

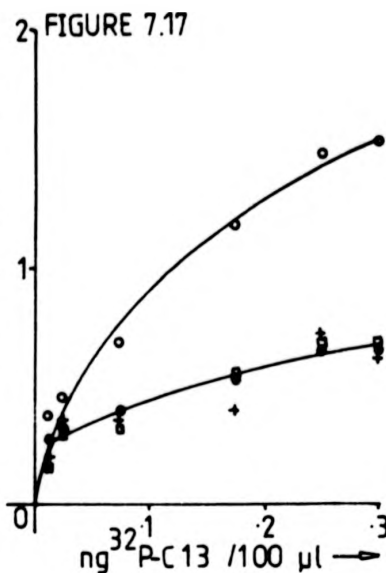
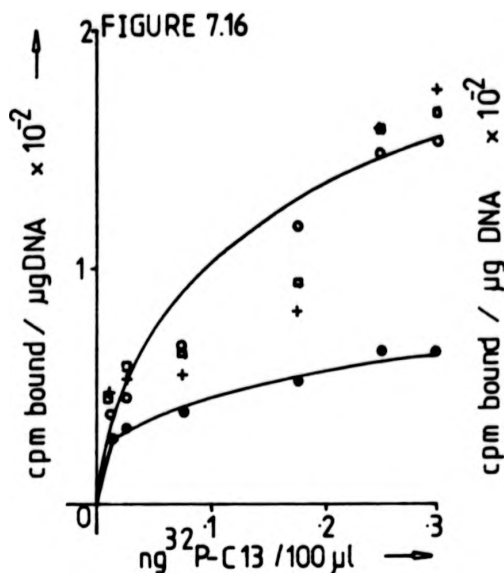
synthesis appears to be the same as in X.laevis (Macleod and Bird, 1982). Thus demethylation cannot be the sole requirement for ribosomal gene transcription, since the erythrocyte nucleus requires more than active RNA polymerase I to transcribe these genes [FIG 3.17]. Whether Fr-18 can only bring about a change in chromatin conformation in demethylated ribosomal genes or in vivo also signals for the demethylation event cannot be decided by using the erythrocyte nuclei.

DNase I Sensitivity of Other Genes.

The oocyte extract, or rather one component from it, has a very specific effect on rRNA transcription. It does this by opening up the ribosomal genes beginning, at the 5' end of the 40S precursor sequence. However, it might have this effect on a variety of genes, which because of their small number or the restrictions of the transcription assay are not detected. Therefore, a series of genes were studied, to see if there was any change in their DNase I sensitivity state on preincubation of the erythrocyte nuclei with an egg or oocyte extract. The DNase I sensitivity of these genes in liver nuclei was also determined.

The genes studied were histone H4 (probe pcX1H4W1, Turner and Woodland, 1982), the oocyte specific 5S RNA (probe Xlo31, Federoff and Brown, 1978), and the transfer RNA genes for methionine 1, phenylalanine, asparagine, alanine, leucine, lysine and tyrosine (probe λ t210, Clarkson et al, 1978; Bryan et al, 1981). These are all transcribed at a high rate during oogenesis. The DNase I sensitivity of the globin genes (probe C13), which is only transcribed in erythropoietic cells was also investigated.





FIGURES 7.16, 7.17

DNase I Sensitivity of the Globin Genes in *X. laevis* Erythrocyte and Liver Nuclei.

Figure 7.16

$^{32}\text{P-C13}$ (1.1×10^7 cpm/ μg) was hybridized to the following DNA samples, as described previously: total DNA from erythrocyte (o---o) and liver nuclei (□---□), as well as DNA from erythrocyte (●---●) and liver (+---+) nuclei that had been digested with DNase I.

Figure 7.17

$^{32}\text{P-C13}$ (1.1×10^7 cpm/ μg) was hybridized to the following DNA samples, as described previously: total DNA (o---o), DNA from erythrocyte nuclei that had been digested with DNase I (●---●) and from erythrocyte nuclei that had been preincubated with an oocyte (□---□) or egg (+---+) extract prior to the DNase I digestion.

DNase I Sensitivity of Genes Transcribed by RNA Polymerase II.

1. Globin.

Globin genes are active in the immature erythrocytes of Xenopus, but not in the adult cells. However, the latter contain RNA polymerase II molecules tightly bound to the chromatin (Hentschell and Tata, 1978) and in avian erythrocytes it has been shown that these are present on the globin genes (Gariglio et al., 1981). In this sense the globin genes could be described as "active", even though their activity is nothing more than a very slow elongation of the final globin mRNA transcript. This correlates with the observation that in avian erythrocytes, globin sequences are preferentially digested by DNase I (Weintraub and Groudine, 1976; Stadler et al., 1980; Bellard et al., 1980). The same is true for the erythrocyte nuclei used here [FIG 7.16], but not for the liver nuclei which do not synthesize globin. The sensitive state of the globin genes in the erythrocyte nucleus is not altered by pre-treatment of the nuclei by either an egg or oocyte extract [FIG 7.17].

2. Histone H4.

Like globin, these genes are transcribed by RNA polymerase II, however, histone H4 synthesis occurs in all actively dividing cells during S phase. Transcription of the histone H4 genes also occurs at a very high rate during oogenesis. So if Fr-18 were having a general effect on all genes transcribed during oogenesis, it should change the DNase I sensitivity state of the histone H4 genes. When the DNase I sensitivity of the histone H4 genes was determined for both types of nuclei [FIG 7.18] it could

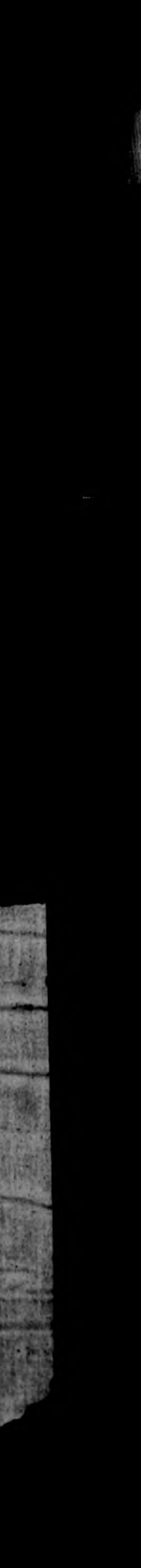
DNase I Sensitivity of Genes Transcribed by RNA Polymerase II.

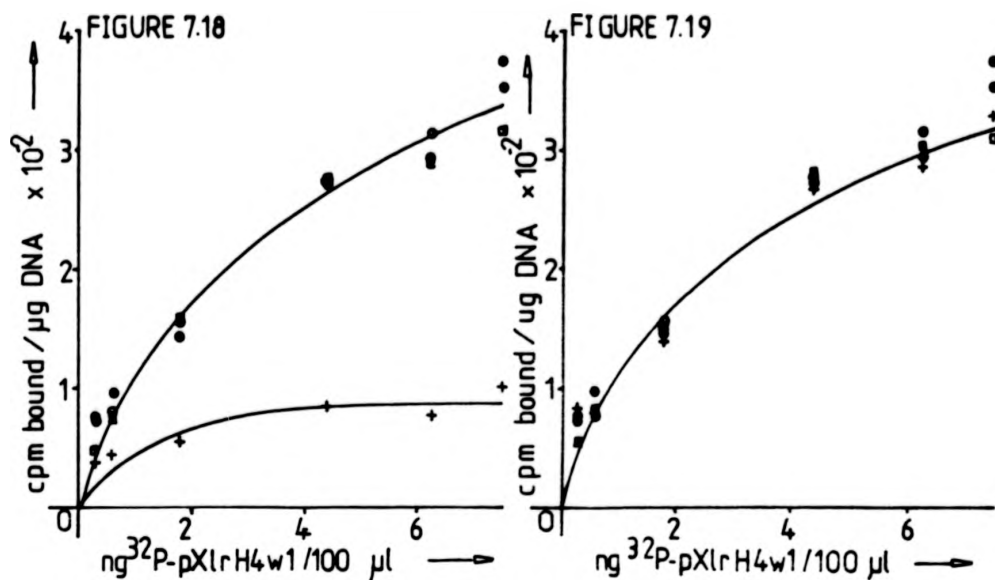
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Like globin, these genes are transcribed by RNA polymerase II, however, histone H4 synthesis occurs in all actively dividing cells during S phase. Transcription of the histone H4 genes also occurs at a very high rate during oogenesis. So if Fr-18 were having a general effect on all genes transcribed during oogenesis, it should change the DNase I sensitivity state of the histone H4 genes. When the DNase I sensitivity of the histone H4 genes was determined for both types of nuclei [FIG 7.18] it could





FIGURES 7.18, 7.19

DNase I Sensitivity of the Histone H4 Genes in *X.laevis*
Erythrocyte and Liver Nuclei.

Figure 7.18

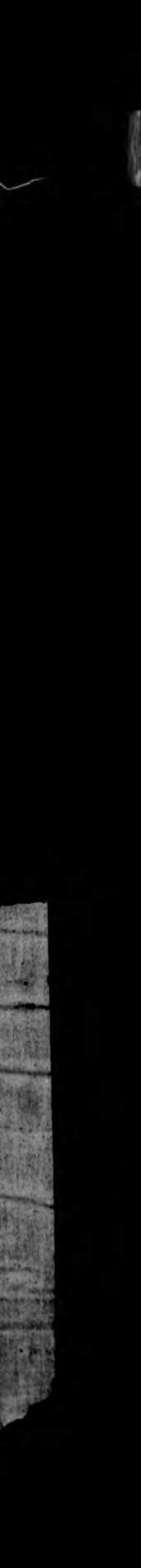
³²P-pcX1H4W1 (1.6×10^7 cpm/ μ g) was hybridized to the following DNA samples, as described previously: total DNA from erythrocyte (o---o) and liver nuclei (□---□), as well as DNA from erythrocyte (●---●) and liver (+---+) nuclei that had been digested with DNase I.

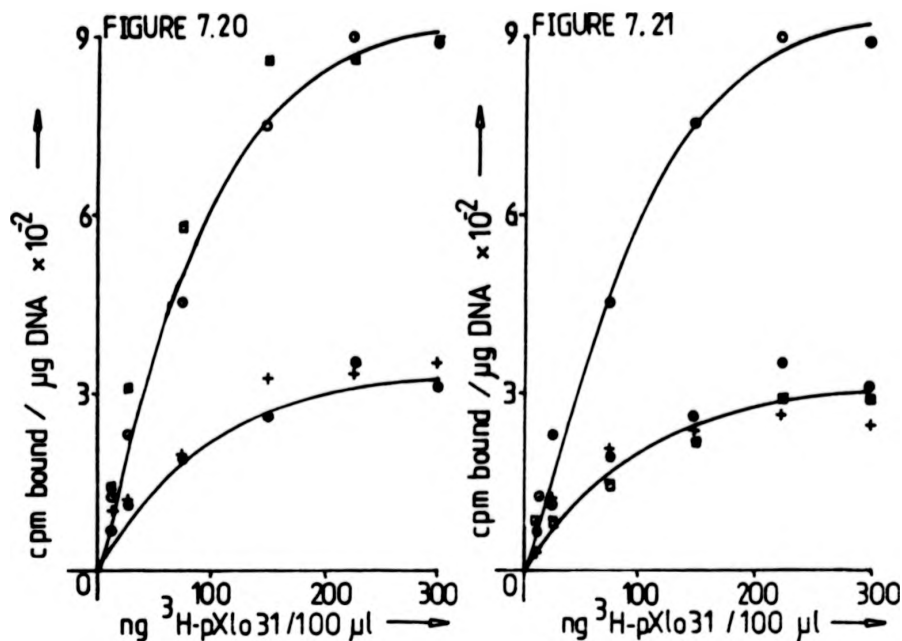
Figure 7.19

³²P-pcX1H4W1 (1.6×10^7 cpm/ μ g) was hybridized to the following DNA samples, as described previously: total DNA (o---o), DNA from erythrocyte nuclei that had been digested with DNase I (●---●) and from erythrocyte nuclei that had been preincubated with an oocyte (□---□) or egg (+---+) extract prior to the DNase I digestion.

be seen that the histone H4 genes are insensitive to DNase I in the erythrocyte nuclei and sensitive in the liver nuclei. Insensitivity in the erythrocyte is expected, since these cells have abandoned division. Although liver cells can be stimulated to rapid division by partial hepatectomy, little division occurs in the normal adult liver from which the nuclei were derived. However, whether there is no histone mRNA synthesis in liver and other non-S phase cells has yet to be determined. Pre-treatment with an oocyte extract does not alter the insensitive state of erythrocyte histone H4 genes [FIG 7.19]. The oocyte extract used was a whole ovary preparation and therefore contained oocytes at all stages of development. Histone mRNA is synthesized in pre-vitellogenic oocytes (Darnborough and Ford, 1976; Ruderman and Pardue, 1977; van Dongen et al, 1981), but further synthesis may occur later on during oogenesis (Anderson and Smith, 1978). Preincubating the erythrocyte nuclei with an egg extract also does not have any effect on the DNase I sensitivity of these genes.

The crude approach of DNase I solubilization is capable of detecting different DNase I sensitivities of genes active in one cell type and inactive in another, as well as different genes with different activities in the same cell type. An oocyte extract made from oocytes at all stages of oogenesis has no effect on modifying the DNase I sensitivity of the histone H4 genes, even though histone mRNA production is a major feature of oogenesis. Neither has it an effect on desensitizing the active globin genes of the erythrocyte nucleus. It would appear that the effect of the oocyte extract is confined to the ribosomal RNA genes on the basis of these hybridizations.





FIGURES 7.20, 7.21

DNase I Sensitivity of the 5S oocyte RNA Genes in *X. laevis* Erythrocyte and Liver Nuclei.

Figure 7.20

$^3\text{H-pX1031}$ (5.5×10^5 cpm/ μg) was hybridized to the following DNA samples, as described previously: total DNA from erythrocyte (o---o) and liver nuclei (□---□), as well as DNA from erythrocyte (●---●) and liver (+---+) nuclei that had been digested with DNase I.

Figure 7.21

$^3\text{H-pX1031}$ (5.5×10^5 cpm/ μg) was hybridized to the following DNA samples, as described previously: total DNA (o---o), DNA from erythrocyte nuclei that had been digested with DNase I (●---●) and from erythrocyte nuclei that had been preincubated with an oocyte (□---□) or egg (+---+) extract prior to the DNase I digestion.

The DNase I Sensitivity of Genes Transcribed by RNA Polymerase

III.

1. 5Sooc RNA.

There are two types of 5S ribosomal RNA genes in Xenopus laevis, the oocyte specific (5Sooc) and somatic (5Ssom) genes (Wegnez et al, 1972; Ford and Southern, 1973). The 5Sooc genes are a family of about 20,000 genes per haploid genome (Brown et al, 1971), interspersed with an equal number of pseudogenes, the latter representing residues 1-101 of the normal 120 bp gene (Jacq et al, 1977). Transcripts of this pseudogene in vivo have never been detected, but their occurrence remains a possibility (Miller and Melton, 1981). The 5Sooc genes are transcribed exclusively during oogenesis, whilst in other cells it is the much smaller number (400 copies per haploid genome; (Peterson et al, 1980) of 5Ssom genes which is used (Wegnez et al, 1972; Ford and Southern, 1973). This number is far too small for the somatic genes to be detected by the straightforward hybridization experiments used here. However, calculation of a rough gene number from Figure 7.20 (21,262 +/- 1000 copies per haploid genome) confirms that the 5Sooc genes are being measured in these hybridizations. This calculation is based on a double reciprocal plot of the data in Figure 7.20 ($y=0.00061+0.09057x$) and



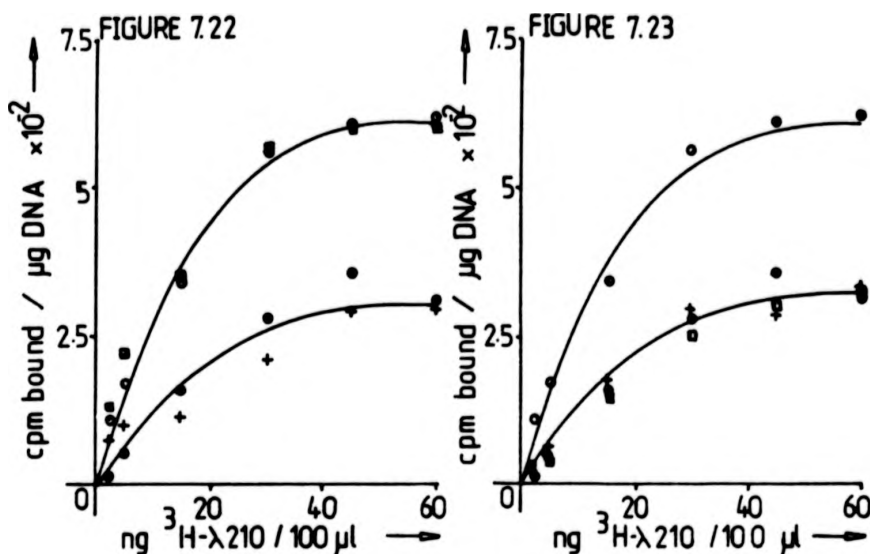
on the assumption that the probe used has plasmid tails on the insert of 50 bp. If the plasmid was not degraded into smaller fragments but remains intact during the nick-translation (unlikely since DNase I was used in the reaction mix) a value of 11,000 +/- 1000 copies per haploid genome is calculated. It can be seen both from Figure 7.20 and Table 7.2 that the 5Sooc genes are sensitive to DNase I in both liver and erythrocyte nuclei. No 5S genes are transcribed in erythrocytes, which have lost all of their RNA polymerase III molecules during maturation (Hentschell and Tata, 1978), nor are the 5Sooc genes believed to be transcribed by liver nuclei (Korn and Gurdon, 1981), where the 5Ssom genes should provide all of the 5S rRNA required. Maternal 5Sooc genes would not have been active since oogenesis, a period of at least 2 years. In the case of the paternal 5Sooc genes, it is not known if they were ever active during gametogenesis, but this seems unlikely, since the accumulation of vast numbers of ribosomes, transcribed in part from the 5Sooc genes, is a feature of oogenesis and not spermatogenesis. Thus this half of the 5Sooc gene complement has probably been inactive for a minimum of 4 years (2 life cycles).

Incubation of the erythrocyte nuclei with an oocyte or an egg extract before DNase I digestion [FIG 7.21] neither increases nor decreases the degree of DNase I sensitivity of the 5Sooc genes.

2. tRNA genes

As seen for the 5Sooc genes, the tRNA genes studied (tRNA Met1, Phe, Tyr, Asn, Ala, Leu, Lys) are equally sensitive to DNase I in erythrocyte and liver nuclei [FIG 7.22]. This is





FIGURES 7.22. 7.23

DNase I Sensitivity of a selection of tRNA Genes in *X. laevis*
Erythrocyte and Liver Nuclei.

Figure 7.22

$^3\text{H}-\lambda\text{t}210$ (3.9×10^6 cpm/ug) was hybridized to the following DNA samples, as described previously: total DNA from erythrocyte (o---o) and liver nuclei (□---□), as well as DNA from erythrocyte (●---●) and liver (+---+) nuclei that had been digested with DNase I.

Figure 7.23

$^3\text{H}-\lambda\text{t}210$ (3.9×10^6 cpm/ug) was hybridized to the following DNA samples, as described previously: total DNA (o---o), DNA from erythrocyte nuclei that had been digested with DNase I (●---●) and from erythrocyte nuclei that had been preincubated with an oocyte (□---□) or egg (+---+) extract prior to the DNase I digestion.

TABLE 7.2

DNase I Sensitivity of Various Genes in Erythrocyte and Liver Nuclei.

DNA Source	Percentage binding of probe;				
	rDNA	Globin	Histone H4	5Sooc	tRNA
Total undigested	100	100	100	100	100
Erythrocyte Nuclei					
x DNase I	100	42.5	100	34.9	51.0
Liver Nuclei					
x DNase I	54.5	100	28.5	39.5	47.8

These data are calculated from the cpm bound/ μ g nuclear DNA at the maximum amount of probe used for each experiment. as shown in Figures 7.3, 7.16, 7.18, 7.20, 7.22.

despite the fact that, although the tRNA genes are transcribed in liver nuclei, they are not transcribed in erythrocyte nuclei. The RNA polymerase III molecules are lost from the maturing erythrocyte (Hentschell and Tata, 1978). Like the 5Soc genes, these tRNA genes do not alter their DNase I sensitive state when the erythrocyte nuclei are pre-treated with either an egg or an oocyte extract [FIG 7.23].

Thus the genes transcribed by RNA polymerase III seem to be DNase I sensitive regardless of their transcriptional activity. This is not due to an artefact in the preparation of the nuclei since the other genes studied, rRNA, histone H4 and globin, do not show unexpectedly high DNase I sensitivity [TABLE 7.2]. It could be argued that any gene that has been transcribed at a given stage of the life cycle of an organism remains DNase I sensitive, even if the gene is not transcribed later (Weintraub and Groudine, 1976; Young et al, 1978). This argument could be used to explain the sensitivity of the tRNA genes in the erythrocyte nuclei. However, it is harder to sustain this argument in the case of the 5Soc genes, which show a similar DNase I sensitivity. It would mean that all the genes ever active in oocytes (and this would probably amount to about 15% of the DNA; Davidson, 1976) are always in the sensitive state, even when they pass through the male for one or more generations (as 50% of the 5Soc genes have done). The insensitivity of the histone H4 and ribosomal RNA genes in the erythrocyte nuclei is also inconsistent with this idea, since these genes must have been active in erythroblasts. It seems more likely that the genes transcribed by RNA polymerase III have a different structure from



other genes, at least in their inactive state, with the result that they are always DNase I sensitive. One possibility is that the difference simply relates to their very small size and tandem repetition. A number of genes transcribed by RNA polymerase II have been shown to have sites hypersensitive to DNase I associated with them (Kuo et al., 1979; Wu, 1980; Keane et al., 1981; Samal et al., 1981), and for some genes these sites are always sensitive, regardless of the activity of the genes (Wu, 1980). In genes which are not tandemly repeated, or where the repeat length is several kb long, cutting these sites does not release the gene from the nucleus. The whole 5S_{00c} gene tandem repeat, on the other hand, is only about 700 bp in Xenopus laevis, and should a hypersensitive site exist, the small fragment of chromatin might leave the nucleus, even if its microstructure was like that of larger inactive genes. If this is the explanation of my results there would have to be hypersensitive sites present in inactive RNA polymerase III genes, just as in the Drosophila heat shock protein genes (Wu, 1980) and histone genes (Samal et al., 1981). The position of the 5S genes in Xenopus could also add to their ease of digestion by DNase I and subsequent loss from the nucleus. Location at the ends of the chromosomes (Pardue et al., 1973) might make them more vulnerable to digestion by DNase I. This, however, does not explain the DNase I sensitivity of the tRNA genes which are scattered throughout the genome. (Pardue et al., 1973) Whatever the reason for the DNase I sensitivity of these RNA polymerase III transcribed genes, in their inactive, non-transcribed state, unlike the RNA polymerase I and II transcribed genes, the DNase I solubilization cannot be taken as an indication of the activity of these genes.

The component of the oocyte extract that brings about RNA polymerase I transcription in erythrocyte nuclei acts only on the ribosomal genes. Other genes, eg histone H4, that are also transcribed at a high rate during oogenesis are not affected. Neither are the globin genes which would not be transcribed during oogenesis or even early embryogenesis. The 5S and tRNA genes though, do not seem to conform to the simple DNase I sensitive-active gene rule, so any effect that an oocyte extract might be having on these genes cannot be seen. The change in chromatin conformation of the ribosomal genes, presumably starting at the 5' end of the transcribed region, allows for accurate transcription of the gene. This transcription itself might keep the rest of the gene in a more relaxed state, or a wave of change to a different chromatin configuration might occur, starting at the 5' end of the gene and moving along the chromatin. This wave, since Fr-18 on its own has only an effect on the beginning of the gene, may be set up by other non-specific oocyte and egg components, which would require Fr-18 for specificity and direction. In this case, the second component cannot be bringing about a wave of demethylation along the gene. The areas of undermethylation in the ribosomal gene repeat are located within the non-transcribed spacer, not the transcribed region (Bird et al, 1981) and the erythrocyte rDNA is already undermethylated. Erythrocyte rDNA is also DNase I insensitive so it is not the demethylation event that causes this change from DNase I resistant to DNase I sensitive. Newly synthesized rRNA is not seen until the mid-late blastula stage of embryogenesis (Shiokawa et al, 1981a, b), no rRNA transcripts can be seen in the very earliest stages. Bird et al (1981) have shown that during this time in X. laevis, but not in the

closely related species X.borealis (Macleod and Bird, 1982), there is a shift towards the ribosomal genes becoming undermethylated at the two specific sites which parallels the appearance of the new rRNA. This is also paralleled by the appearance of the "oocyte" component that brings about specific rRNA synthesis in erythrocyte nuclei. So it might be expected that the rDNA from early cleavage embryos is DNase I resistant, and that from neurula stage embryos is DNase I sensitive. It is not possible to say if the demethylation event comes first and acts as a signal to the Fr-18, or the other way round, or in the light of more recent work (Macleod and Bird, 1982) it may well be that the demethylation event has nothing to do with rRNA gene control. Fr-18 certainly acts on undermethylated chromatin. To see if it has any effect on methylated rDNA, the assays would have to be repeated using early cleavage embryo or sperm nuclei.

CHAPTER VIII; CONCLUSIONS.

The erythrocyte nuclei of Xenopus laevis are transcriptionally inactive, most of the small amount of radioactive precursor incorporated when they are placed in a transcription assay is due to the elongation of the final RNA polymerase II transcripts. Although some RNA polymerase III transcription is measured, it is at a very low level. RNA polymerase I activity is not seen, these molecules are lost during erythrocyte maturation (Hentschell and Tata, 1978) and are not sequestered in the nucleus as tightly bound transcriptional complexes. This is in marked contrast to the amount of RNA synthesis seen during oogenesis. In oocytes, transcription proceeds rapidly, at least in part to provide the RNA species needed for the early division stages of embryogenesis and beyond. Enough ribosomes are produced to last the developing embryo to the swimming tadpole stage (Brown and Gurdon, 1964), amounting to 4 μ g rRNA (Perkowska et al, 1968) which is transcribed by the vitellogenic oocyte.

By mixing the inactive rRNA genes of an erythrocyte nucleus and the soluble proteins from an oocyte it has been possible to induce these inactive genes to be transcribed. This transcription involves new initiation by the RNA polymerase I molecules. The transcription of the rRNA genes is not simply a reflection of the presence of RNA polymerase I molecules in the oocyte extract, since RNA polymerase I molecules on their own do not transcribe rDNA in the erythrocyte nuclei. A soluble protein extract from unfertilized eggs, although containing comparable amounts of RNA polymerase, does not elicit transcription by RNA polymerase I when mixed with erythrocyte nuclei. This parallels the lack of

transcription seen in unfertilized eggs. Presumably the egg extract lacks the oocyte component that brings about RNA polymerase I activity on inactive rDNA chromatin. There is a good correlation between the ability of an oocyte, egg or embryo extract to do this and the activity of the ribosomal genes in the cells from which the extract is made. Previtellogenic oocyte extracts, early cleavage and blastula embryo extracts are unable to induce RNA polymerase I to transcribe erythrocyte rDNA, whilst vitellogenic oocyte extracts and gastrula and later stage embryo extracts can do so. In all extracts the amount of RNA polymerase I remains the same. It is a reasonable proposition that the reason small oocytes, eggs and cleaving embryos fail to make rRNA is the same as the failure of their extracts to induce it in erythrocyte nuclei. If this is so rRNA transcription is controlled by an activating molecule or molecules that are not detectable in young oocytes, unfertilized eggs and early cleavage embryos. Unfortunately another possibility is that the stimulating molecule is present in the inactive cells and is lost during extraction.

Column chromatography of the oocyte extract revealed a single peak of stimulatory activity both on Sephadex G100 and DEAE cellulose columns. This is as a result of a small protein (molecular weight about 21000 daltons) that is heat and trypsin sensitive. This peak, termed Fr-18, is found in all protein extracts that bring about RNA polymerase I transcription in the erythrocyte nuclear assay. Although RNA polymerase I was used to identify and locate Fr-18, it appears that Fr-18 acts independently of the RNA polymerase, by associating with the nuclei during the preincubation.

By studying the chromatin conformation of the erythrocyte rDNA using DNase I, it has been shown that incubating the nuclei with an oocyte extract, but not an egg extract, causes the DNase I resistant rDNA to become DNase I sensitive. During the induction of rRNA synthesis the entire rRNA gene repeat, both transcribed and non-transcribed sequences, become DNase I sensitive, as is also the case if the erythrocyte nuclei are preincubated with an egg extract and Fr-18. Since no change is seen in any of the other genes investigated, the alteration in conformation must be limited to the rRNA genes. Fr-18 interacts with the beginning of the rRNA gene, at the external transcribed spacer, or just beyond it. The site might be co-extensive with the rRNA promoter site (Moss, 1982), but no effect is seen on the two reduplicated promoters (Moss and Birnstiel, 1979), located in the non-transcribed spacer. There is no evidence for a DNase I hypersensitive site at any point in the rRNA gene repeat, so Fr-18 cannot be interacting with, or producing such a structure. Alone it only causes a small region of the rRNA gene to become DNase I sensitive. If the nuclei are preincubated with an oocyte extract and Actinomycin D such that the RNA polymerase I molecules can initiate, but not elongate, more of the gene becomes DNase I sensitive, but only the transcribed sequences and not the non-transcribed spacer.

Therefore Fr-18 would appear to act on a specific site at about the beginning of the transcribed sequence, causing a shift to a DNase I sensitive state. Exactly how this is achieved is not clear. The oocyte extract contains more than just Fr-18 and RNA polymerases, so some other specific or non-specific factor could be necessary to propagate the DNase I sensitive state from the start given by Fr-18. This factor would have to be present in the

egg extract even though alone it cannot induce rRNA synthesis. Since the crude RNA polymerase preparation can substitute for egg extract in inducing rRNA synthesis, the factor is also likely to be present here. However, I have not shown that the crude RNA polymerase preparation plus Fr-18 changes the conformation of erythrocyte rDNA.

Further experiments to determine how Fr-18 interacts with the rDNA need to be carried out. In particular, whether Fr-18 binds to the rDNA and, if it does so, to which particular sequence. The mode of action of the change to a DNase I sensitive state also requires further investigation, especially the interaction of Fr-18 with the non-specific factor (which also should be identified) necessary for full DNase I sensitivity. It could also be possible to set up a X.laevis RNA polymerase I transcription system using the rDNA plasmids, to determine whether Fr-18 directs RNA polymerase I under these circumstances. Fr-18 should ideally be further purified, perhaps by raising a monoclonal antibody and using this both for further purification and studying the location of Fr-18 within the oocyte nucleus using fluorescent techniques. This should also reveal whether there are any differences between the oocyte active component and that found in neurula embryos.

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Since the initial discovery that the DNase I sensitivity of the globin genes in different cell types correlates with globin gene expression¹, this relationship has been shown to hold true for a variety of genes, including the genes for ovalbumin², conalbumin³, α - and β -globin in chicken^{4,5}, several heat-shock proteins in *Drosophila*^{6,7}, the r-chromatin of *Tetrahymena*⁸ and the viral polyoma minichromosome⁹. Although genes transcribed by RNA polymerases I and II have been studied extensively, the genes transcribed by RNA polymerase III have not. We have therefore investigated the DNase I sensitivity of transfer RNA (tRNA) and oogenetic 5S RNA genes in the liver and erythrocyte nuclei of *Xenopus laevis*. The oogenetic 5S genes are not transcribed in any known somatic cell^{10,11}, and tRNA genes are transcribed in the hepatocyte but are inactive in the erythrocyte. We show here that, although in these two cell types the correspondence between DNase I sensitivity and gene transcription holds good for globin and the ribosomal genes, the tRNA and oogenetic 5S genes are DNase I sensitive in both liver and erythrocyte nuclei. Thus for the genes transcribed by polymerase III the correspondence of sensitivity and expression breaks down.

There are two types of 5S ribosomal gene in *X. laevis*: oocyte specific ($5S_{\text{oc}}$) and somatic ($5S_{\text{sm}}$)^{10,11}. The $5S_{\text{oc}}$ genes are a family of about 20,000 genes per haploid genome¹², interspersed with an equal number of 5S pseudogenes, representing residues 1-101 of the normal 120 bp gene¹³. Transcripts of the pseudogene *in vitro* have never been detected, but their occurrence remains a possibility¹⁴. The $5S_{\text{sm}}$ genes are transcribed exclusively during oogenesis, whilst in other cells it is the $5S_{\text{oc}}$ genes (of which there are fewer, about 400)¹⁵ which are used^{10,11}. This number is far too small for the somatic genes to be detected by the straightforward hybridization experiments described here. A rough calculation of gene number from Fig. 1a (11,000-21,000 copies of the gene per pseudogene repeat per haploid genome, depending on the amount of vector sequences involved in the hybridization) confirms that, in effect,

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Equally surprising is the finding (Fig. 1b) that certain of the tRNA genes, which are transcribed in the hepatocyte but not in the erythrocyte, are equally sensitive in both types of nucleus. The probe used contains two tRNA^{Met} genes and one each of the tRNAs accepting phenylalanine, tryptophan, asparagine, alanine, leucine and lysine¹⁸. The complete 3.18 kilobase (kb) unit is repeated about 100 times per haploid genome¹⁹. The probe probably reveals all of these tRNA species present in the genome, but some of them may be pseudogenes and never expressed. Even if this were the case, it would not alter the conclusion that these tRNA genes are hypersensitive to DNase I, even when they are not expressed.

Thus in blood and liver cells, many, and perhaps all, genes transcribed by polymerase III seem to be DNase I sensitive, regardless of their transcriptional activity. Maternal $5S_{\text{oc}}$ genes have not been active since oogenesis, a period of at least 2 yr. In the case of the paternal $5S_{\text{oc}}$ genes, it is not known whether they were even active during gametogenesis, but this seems unlikely as the accumulation of vast numbers of ribosomes, transcribed in part from the $5S_{\text{oc}}$ genes, which is a feature of oogenesis, does not occur during spermatogenesis. Thus this half of the $5S_{\text{oc}}$ gene complement may have been inactive for as much as 4 yr (two life cycles).

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It might be suspected that these results reflect an artefact in the preparation and digestion of the nuclei, and therefore that many other genes would also show unexpectedly high nuclease sensitivity. However, measurement of the concentrations of several other genes in the same DNA samples yielded the predicted results. For example, ribosomal genes are not transcribed in erythrocytes¹⁶, nor is RNA polymerase I present in their nuclei, but the opposite is true of liver. As expected the ribosomal DNA from liver was more sensitive to DNase I digestion than bulk DNA, but equally resistant in erythrocytes (Fig. 2a). Globin genes are active in the immature erythrocyte of *Xenopus*, but not in the adult cell. The latter, however, contains RNA polymerase II molecules tightly bound to the chromatin¹⁶ and in avian erythrocytes the frozen polymerases are to be found on the globin genes¹⁷. In this sense the globin genes could be regarded as 'active genes', which correlates with the observation that in avian erythrocytes, globin sequences are preferentially digested by DNase I^{4,5}. The same is the case in human erythrocytes (Fig. 2b), but not hepatocytes, which do not synthesize globin. Our results confirm those of others¹⁷, and show that the crude approach of DNase I solubilization is capable of detecting different DNase I sensitivity in genes active in one cell type and inactive in another.

The results of Figs 1 and 2 show a single time point of digestion and leave open the possibility that the inactive tRNA or 5S genes show different kinetics of digestion from active genes. Figure 3 shows that this is not the case; in fact, 5S_{liver} genes demonstrate even greater sensitivity to DNase I than do the active ribosomal genes of liver.

It has been argued^{12,13} that any gene that has been transcribed at a given stage of the life cycle of an organism remains DNase I sensitive, even if the gene becomes inactive later. This argu-

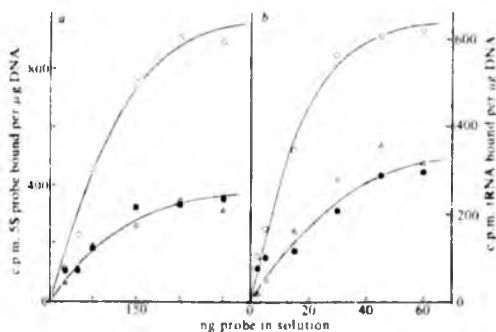


Fig. 1 DNase I sensitivity of 5S (a) and tRNA (b) genes in erythrocyte and liver nuclei. Erythrocyte nuclei were prepared according to the method of ref. 26 and the liver nuclei by the method of Scribner and Weber²⁷. Nuclei from the two tissues were digested with pancreatic DNase I (20 µg ml⁻¹) for 5 min, which is sufficient to release 5–10% of the DNA from the nuclei. The nuclei were pelleted by centrifugation for a few seconds in an Eppendorf microfuge and their DNA extracted according to the method of Weintraub and Groudine¹. The alkali-denatured DNA was loaded onto nitrocellulose filters²⁸ and hybridized with nick-translated recombinant DNA²⁹ containing the relevant gene sequences. This procedure will not detect a hypersensitive site³⁰ in the way that a blot-transfer hybridization of electrophoresed DNA would, but it gives a more quantitative assay of the DNase I sensitivity of the genes as a whole¹. The 5S_{liver} probe used was pXlo31 which contains a single repeat of the 5S gene plus a pseudogene³¹. It was labelled with ³²P-dCTP to a specific activity of 5.5×10^5 c.p.m. µg⁻¹. The tRNA probe used was t210, which contains two copies of tRNA^{Met} and one copy each of tRNA^{Pro}, tRNA^{Leu}, tRNA^{Val}, tRNA^{Ala}, tRNA^{Arg} and tRNA^{Tyr} (refs 18, 19). It was labelled with ³²P-dCTP to a specific activity of 3.9×10^6 c.p.m. µg⁻¹. Undigested liver DNA gave a result identical to that for erythrocyte, so the data are not shown. ○, Undigested erythrocyte DNA; △, digested erythrocyte DNA; ●, digested liver DNA.

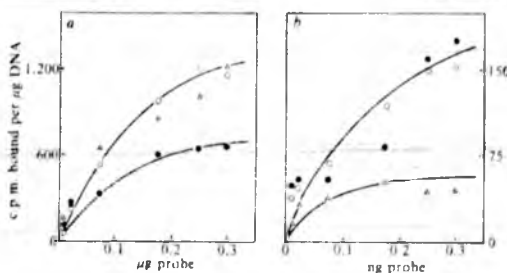


Fig. 2 DNase I sensitivity of ribosomal RNA (a) and globin (b) genes in erythrocyte and liver nuclei. The DNA samples and procedures were as for Fig. 1. The ribosomal probe used was pXlr101, constructed by Dr R. Reeder; it contains a complete repeat of the 40S precursor plus non-transcribed spacer. It was nick-translated with ³²P-dCTP to 2.2×10^5 c.p.m. µg⁻¹. The globin probe was labelled with ³²P-dCTP to 1.1×10^5 c.p.m. µg⁻¹. It was a gift from Dr R. W. Old, and contains an ~300 bp sequence complementary to a globin mRNA. ○, Undigested erythrocyte DNA (liver gave identical results); △, DNase I-digested erythrocyte DNA; ●, DNase I-digested liver DNA.

ment could be used to explain the sensitivity of the tRNA genes in the erythrocyte nuclei. It is harder, however, to sustain this argument in the case of the 5S_{liver} genes, which show a similar DNase I sensitivity. It would mean that any gene active in oocytes (and this would probably amount to ~15% of the DNA²²) would always be in the sensitive state, even when it passed through the male for one or more generations (as 50% of the 5S_{liver} genes have done). The insensitivity of ribosomal genes in the erythrocyte nuclei is also inconsistent with this idea, since these genes must have been active in erythroblasts. It seems more likely that the genes transcribed by RNA polymerase III have a different structure from other genes, at least in their inactive state, with the result that they are always DNase I sensitive. The difference might simply relate to their very small size and tandem repetition. Several RNA polymerase

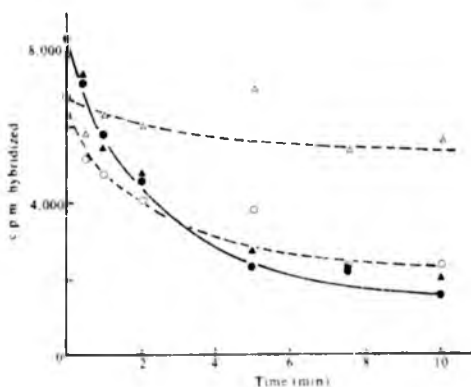


Fig. 3 Time course of DNase I digestion. Erythrocyte and liver nuclei were digested with 20 µg ml⁻¹ DNase I for increasing periods of time, the reaction was stopped by the addition of SDS and EDTA to a final concentration of 0.5% SDS, 12.5 mM EDTA, and the DNA extracted as previously described. One µg DNA from each time point was loaded onto duplicate nitrocellulose filters, which were hybridized in a final volume of 200 µl with either 250 ng ³²P-Xlr101 or 200 ng ³²P-Xlo8 for 24 h before washing and counting. pXlr101 and pXlo8 were labelled with ³²P-dCTP to a specific activity of 5.6×10^5 and 3.6×10^5 c.p.m. µg⁻¹ respectively. pXlo8 contains three copies of the gene-pseudogene 5S repeat³². Further details are given in Fig. 1 legend. Erythrocyte ribosomal genes, ○, liver ribosomal genes; △, erythrocyte 5S genes; ●, liver 5S genes.

II-transcribed genes have been shown to be associated with sites hypersensitive to DNase I^{19,22} and for some genes these sites are always sensitive, regardless of the activity of the genes²³. For genes in which small units are tandemly repeated this might be a serious problem. We have therefore examined the 5S_{oic} repeat unit by digesting the DNA samples of Fig. 3 with the restriction enzyme *Hind*III, running the digested DNAs on agarose gels, transferring them to nitrocellulose and probing with pX1031. *Hind*III cuts once within the 5S_{oic} gene/pseudogene repeat, yielding a 700 bp fragment. If a hypersensitive site were present it would act like another restriction site, giving a defined fragment or fragments smaller than 700 bp. The 700 bp repeat disappeared with the expected

kinetics and without revealing discrete smaller fragments (data not shown). Thus it does not seem that our results can be explained by the presence of specific hypersensitive sites. Another possible explanation of the 5S_{oic} sensitivity is that the location of these genes at the telomeres of several chromosomes²⁴ confers special nuclease sensitivity on them. This possibility could be tested by studying a species, such as *Rana temporaria*, in which the 5S genes are not telomeric²⁵. In any case, the tRNA repeat studied here does not have a telomeric location. (See G. Fostel, M. L. Pardue and S. G. Clarkson, quoted in ref. 18.) Whatever the reason for the phenomenon, it appears that ready DNase I solubilization cannot be used as an indication of activity in genes transcribed by polymerase III.

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Is avian adeno-associated virus an endogenous virus of chicken cells?

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The adeno-associated viruses (AAV) are defective parvoviruses which produce infective progeny only in cells co-infected with a 'helper' adenovirus (Ad)¹. Both human and simian AAV have been recovered from human and simian primary cell cultures following their inoculation with 'AAV-free' Ad. Whereas some studies have suggested that AAV exists in a latent state in these cells^{2,3}, others have indicated that the AAV genome is capable of establishing and maintaining a latent state in defined laboratory conditions⁴⁻⁷ which mimic the situation proposed for the 'latent' AAV recovered from human and simian tissues. Here, avian adeno-associated virus (AAAV) was consistently recovered from limiting dilutions of purified and unpurified avian Ad stocks propagated in embryonating chicken eggs derived from two independently raised flocks of White Leghorn (WL) chickens but not when these Ad stocks were propagated in duck cells. These observations suggest that AAAV is a latent endogenous virus of at least some flocks of WL chickens.

Infectious AAV has been vertically transmitted *in utero* in both naturally occurring and experimentally induced infections of hens^{8,9} and in experimentally infected mice¹⁰. An advantage of using chickens for virological studies is the availability of specific-pathogen-free (SPF) embryonating eggs derived from WL chickens which had not been exposed to avian Ad or to AAAV. By using SPF embryos, the possibility of an *in utero* infection with either infectious Ad or AAAV was eliminated. Antibodies to avian Ad or AAAV were not detected either in the SPF chickens during serological surveys conducted by the producer or in the yolks of SPF embryos we tested using the virus neutralization test and the enzyme-linked immunosorbent

assay¹¹. (We had previously demonstrated that yolk antibody levels to avian Ad and AAAV were similar or identical to those present in hens experimentally infected with these viruses¹².)

Two avian Ad strains, CELO virus (serotype 1) and Irish strain 506 (serotype 4), were treated with antiserum to AAAV and inoculated in chicken embryo kidney (CEK) cells prepared from SPF embryos. Various dilutions of the cell lysate were inoculated into sets of 10-day-old embryonating eggs. The allantoamniotic fluids were collected and tested for AAAV as described in Table 1, legend. AAAV was detected in all inoculated eggs by the second passage, even though these Ad stocks were pretreated with AAAV antiserum of high titre. Because our antiserum may not have neutralized all of the AAAV, the CELO virus stock was purified as indicated (Table 1, experiment 2). Three successive isopycnic centrifugations were performed^{12,13} to separate possible contaminating AAAV (buoyant density 1.38 and 1.42 g cm⁻³) from CELO virus (buoyant density 1.34 g cm⁻³). AAAV was not detected when this purified stock was examined by electron microscopy. Two thousand CELO virus particles were counted without detecting a single AAAV particle. However, AAAV was detected in all embryos infected with purified CELO virus (Table 1, experiment 2). In some cases, the allantoamniotic fluids of eggs inoculated with higher dilutions of CELO virus (for example, eggs inoculated with a 10⁻⁹ dilution of CELO virus) had Ad antigens but not AAAV antigens until the second passage. These results suggested that several cycles of viral replication are needed to produce enough AAAV to be detected by the agar gel precipitation test. Similar results were obtained when the above experiment was repeated using embryonating eggs from a second flock of WL hens housed in isolation at the University of Rhode Island Experimental Farm.

Our findings were of great concern because the usual means of assaying AAAV involved inoculating embryonating eggs with CELO virus and various dilutions of AAAV. In previous studies the incubation period was limited to 96 h when the CELO virus inoculum was 10⁴ plaque-forming units (PFU) per ml. AAAV was only detected when added to this system; eggs inoculated with CELO virus alone did not produce detectable AAAV antigens. In the present study, AAAV was invariably produced in embryonating eggs when either the incubation time

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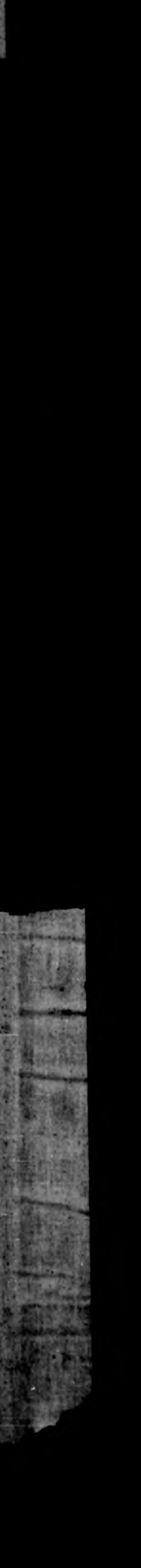
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